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ON ALCOHOL
AND
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ALCOHOLIC LIVER PATHOLOGY

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Preface

This book constitutes the proceedings of the Liver Pathology Section of the International Symposia on Alcohol and Drug Research held in Toronto from October 15 to 18, 1973. These symposia, co-sponsored by the International Council on Alcoholism and Addictions and by the Department of National Health and Welfare, Canada, differed in character and purpose from the symposia held at the various international congresses of scientific disciplines. There are many channels available for the presentation of new scientific information, but relatively few opportunities for researchers to discuss informally and in detail their differences of findings and interpretations.

The organizing committees of the Toronto symposia therefore opted for small closed-group meetings, each one devoted to intensive critical examination of the state of knowledge in a designated area. Despite the regrettable omissions which this decision entailed, it was necessary to restrict the participants and observers at each symposium to a total number sufficiently small to permit free informal discussion. The objective was to encourage the airing of new ideas, the critical appraisal of old ones, and the identification of important directions for future research.

Each symposium was built around a specific problem rather than a specific scientific discipline. One of the hoped-for benefits was the opportunity for specialists in each discipline to see the problem in the broader context provided by the other disciplines bearing on the same problem. This approach is illustrated by the organization of the symposium on liver pathology. The first sessions were devoted to clinical and epidemiological data which might help to sort out the role of alcohol *per se*, as distinct from malnutrition or other factors, in the production of alcoholic liver disease. This was followed by consideration of the liver as a complex organ in a much more complex organism, influenced by blood flow, oxygen supply, hormones, and biochemical events elsewhere in the body. How does alcohol influence the function of the liver within the

body, and of individual hepatocytes within the liver? What actually causes cell death in livers exposed to alcohol in the living subject?

This, in turn led to two other major areas of discussion. The first dealt with ethanol metabolism. Ethanol is both a chemical agent acting on the liver, and a biochemical substrate acted on by the liver. Are these two roles related? Is metabolic alteration in the liver of the alcoholic subject an adaptive process, which permits more efficient utilization of alcohol and protects against its harmful effects? Or does this same "adaptation", by producing imbalance in other metabolic processes, contribute to the pathogenesis of liver disease?

The other major area was that of the sequence of events initiated by cell death, leading to inflammatory and fibrotic response, and culminating in the pattern of irreversible and fatal cirrhosis. What are the histochemical and cytochemical changes accompanying this sequence? Which, if any, are causal and which are merely manifestations of increased reparative activity? The purpose of this session was to clarify the present state of knowledge concerning the factors which determine the transition from reversible to irreversible change.

In the view of the organizing committee and, one hopes, of the participants, the symposium was highly productive. Unfortunately it was not technically possible to record and edit the free discussions, in a form which would be intelligible to the reader, within the time limits set for publication. The committee therefore felt that it would be of greatest value to the potential readers, to concentrate on publishing the formal working papers as rapidly as possible. Most of the papers are, in themselves, wide-ranging critical reviews providing up-to-date information as selected and interpreted by recognized experts in the many aspects of this subject. An attempt has been made to give some reflection of the wide-ranging and stimulating discussion which took place, in the form of Concluding Remarks at the end of the volume.

The organizing committee extends its sincere thanks to the sponsoring organizations, to the support staff of the Addiction Research Foundation who attended to all the essential arrangements, and above all, to the participants who took up so enthusiastically the spirit and purpose of the conference.

PROLOGUE

ODE TO THE LIVER*

*Modest,
organized
friend,
underground
worker,
let me give you
the wing of my song,
the thrust
of the air,
the soaring
of my ode:
it is born
of your invisible
machinery,
it flies
from your tireless
confined mill,
delicate
powerful
entail,
ever alive and dark.
While
the heart resounds and attracts
the music of the mandolin,
there, inside,
you filter
and apportion,
you separate
and divide,
you multiply
and lubricate,
you raise
and gather
the threads and the grams
of life, the final
distillate,
the intimate essences.
Submerged
viscus,
measurer
of the blood,
you live
full of hands
and full of eyes,
measuring and transferring
in your hidden
alchemical
chamber.*

*Yellow
is the matrix
of your red hydraulic flow,
diver
of the most perilous
depths of man,
there forever hidden,
everlasting,
in the factory,
noiseless.
And every
feeling
or impulse
grew in your machinery,
received some drop
of your tireless
elaboration,
to love you added
fire or melancholy,
let one tiny cell
be in error
or one fiber be worn
in your labor
and the pilot flies into the wrong sky,
the tenor collapses in a wheeze,
the astronomer loses a planet.
Up above, how
the bewitching eyes of the rose
and the lips
of the matinal carnation
sparkle!
How the maiden
in the river laughs!
And down below,
the filter and the balance,
the delicate chemistry
of the liver,
the storehouse
of the subtle changes:
no one
sees or celebrates it,
but,
when it ages
or its mortar wastes away,
the eyes of the rose are gone,
the teeth of the carnation wilted
and the maiden silent in the river.*

*Austere portion
or the whole
of myself,
grandfather
of the heart,
generator
of energy:
I sing to you
and I fear you
as though you were judge,
meter,
implacable indicator,
and if I can not
surrender myself in shackles to austerity,
if the surfeit of
delicacies,
or the hereditary wine of my country
dared
to disturb my health
or the equilibrium of my poetry,
from you,
dark monarch,
giver of syrups and of poisons,
regulator of salts,
from you I hope for justice:
I love life: Do not betray me! Work on!
Do not arrest my song.*

Pablo Neruda, 1904-1973
Nobel Laureate in Literature, 1971

Translation by
Oriana Josseu Kalant

Quantitative Aspects of Drinking in Alcoholic Liver Cirrhosis

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The association between chronic alcoholism and cirrhosis of the liver is no longer a matter of controversy. However, a puzzling discrepancy still seems to exist between the high incidence of alcoholism in cirrhosis throughout the world and the apparent rarity of cirrhosis in alcoholics. As autopsy series indicate (especially in studies published after 1945) roughly 50 to 90 per cent of all cases of cirrhosis are due to excessive alcohol consumption in those regions where production and sale of alcoholic beverages is of economic importance. In a recent review of the epidemiology of cirrhosis based on WHO data, Martini and Bode (1970a, 1970b) felt justified in stating that throughout the world 50 per cent of all cases of cirrhosis, if not more, can now be linked to alcohol abuse. In striking contrast to this, cirrhosis is said to be comparatively rare among heavy drinkers, a point which already had been stressed by a number of reputed German pathologists at the turn of the century. From earlier studies (Table I) it can be seen that autopsy records showed the incidence of cirrhosis in alcoholics to range from 2.4 to 28 per cent of the cases. On the basis of 11 series comprising a total of 2045 autopsies, Klatskin (1961) calculated the average incidence of cirrhosis to be 8.7 per cent with a range of 1.2 to 19.1 per cent. This is in agreement with the well known estimate of Jolliffe and Jellinek (1941) that only one out of twelve alcoholics will be found to suffer from cirrhosis of the liver (8.2%). However, it must be borne in mind that varying diagnostic standards, as well as the wide scatter of quantitative aspects to be encountered in any group of labelled alcoholics, are responsible for these differences in frequency. For instance, in many of the older statistics, the figures were based solely on gross examination of livers at autopsy and only the atrophic form of cirrhosis was used as a criterion.

At a later date, investigations based on biopsy material yielded a markedly higher incidence of cirrhosis among alcoholics. Von Oldershausen (1964, 1970) collected 11

TABLE I

INCIDENCE OF CIRRHOSIS OF THE LIVER IN ALCOHOLICS AT AUTOPSY

Author's Name ^a	Year Published	Size of Sample N	Cirrhosis		Comment
			No. of Cases	Per Cent of Sample	
Formad	1886	250	6	2.4	Sudden death due to alcohol.
Klopstock	1906	25	1	4.0	
v. Baumgarten	1908	?	?	5-6	
Barbier a. Jaquis	1939	231	17	7.4	
Delore a. Devant	1939	140	14	10.0	
Fahr	1909/11	309	32	10.4	(13 cases of Laennec's cirrhosis; 19 cases of "fatty liver plus secondary induration")
Kayser	1888	155	21	13.5	Death in delirium tremens
Jagić,	1906	151	22	14.6	Alcohol psychosis
Kern	1913	170	28	16.5	Advanced Laennec's + fatty cirrhosis
			48	28.2	Incipient Laennec's + fatty cirrhosis
Boles a. Clark	1936	228	58	25.4	
Wilens	1947	519	145	27.9	35 years and older; more than 1 pint of whiskey or equivalent per day for many years
		(600)	(40)	(6.7)	(non-alcoholic control group)

^a See References

series of chronic alcoholics from different countries, in whom liver biopsies had been performed and he arrived at an average incidence of cirrhosis of 30.8 per cent. This agrees fully with Leevy's finding (1967a, b, 1968) that in biopsy specimens of 3000 alcoholics admitted over a period of 17 years to a large municipal hospital, varying degrees of cirrhosis of the liver were found in 29 per cent of the cases (Table II).

Though useful for epidemiological purposes, any cross-sectional study of this kind which does not define in quantitative terms what is meant by "alcoholism" will, however, fail to reflect the true association between alcohol and severe liver damage; and it will

prove to be misleading if one attempts to assess the actual risk of cirrhosis in chronic alcoholism.

As early as 1904, Naunyn suspected the influence of a time factor when he stated that "only those among heavy drinkers will be victims of cirrhosis of the liver whom alcohol has not done away with at an earlier age." Von Oldershausen (1964) was the first to demonstrate that in his own sample of 250 alcoholics, the degree of liver damage was clearly correlated to the mean age at which biopsy had been performed. Mean age of cases with normal histology in his group was 37.1 ± 1.9 years ($\bar{x} \pm s_{\bar{x}}$); mean age rose to 58.5 ± 1.1 years for advanced cirrhosis. He concluded that duration of heavy drinking is one of the decisive factors in the development of alcoholic cirrhosis, an opinion again confirmed by recent results as presented by a group of Australian workers (Wilkinson, Santamaria and Rankin, 1969). Von Oldershausen suggested that the surprisingly low percentage of cirrhosis in some of the earlier autopsy statistics might have been due to the age distribution.

TABLE II

INCIDENCE OF CIRRHOSIS OF THE LIVER IN ALCOHOLICS
AS EVIDENCED BY LIVER BIOPSY

Author	Size of Sample	Incidence of Cirrhosis	Comment
von Oldershausen ^a	778	30.8%	Collective data from 11 series
Leevy ^b	3000	29.0%	Alcoholics admitted to a large municipal hospital over a period of 17 years

^avon Oldershausen, 1964, 1970.

^bLeevy and ten Hove, 1967; Leevy, 1967 and 1968.

However, the duration of alcohol influence is only one side of the problem and should be evaluated in conjunction with the average amount of alcohol consumed per time unit. It can be assumed that the high percentage of cirrhosis in Wilens' (1947) autopsy sample, *i.e.* about 28 per cent, was attributable to the fact that cases beyond the age of 35 years were included only if their daily alcohol consumption had exceeded one pint of whiskey or its equivalent for many years; the controls in this group had been either total abstainers or only moderate consumers of alcohol.

There is no doubt that the annual per capita consumption of alcohol in a given country is only a very crude parameter, since the actual frequency distribution presents a highly skewed curve obeying the logarithmic normal law as shown by Ledermann (1956) and later confirmed by de Lint and Schmidt (1965, 1970). Despite this handicap, fluctuations in per capita consumption of alcoholic beverages in the past were seen to be followed by quite identical trends in cirrhosis mortality in the United States, in Canada, and particularly in France where overall consumption of alcohol and death from cirrhosis of the liver reach a maximum. In wine-drinking France a special drinking situation exists since the larger part of the population is accustomed to the daily use of alcohol. The

predominant type of drinking pattern in French alcoholics is that of a continuous "alcohol impregnation" with little overt intoxication. This delta-type of alcoholism seems to be characteristic of drinking habits in viticultural regions. The conspicuous correlation between overall consumption of alcohol and cirrhosis mortality in wine-drinking France gave rise to two carefully planned studies in which alcohol intake and dietary habits of a total of 300 patients suffering from alcoholic cirrhosis were compared with those of 300 non-cirrhotic controls, matched as to age, sex, duration of alcohol use, body weight, physical activity, and social status. The cases were collected from hospitals of five different French towns. These studies which were published by Péquignot (1958, 1961, 1963) showed that for patients with alcoholic cirrhosis average intake had ranged between 170 and 210 grams of ethanol per day for a period of roughly 20 to 25 years, as compared to a daily consumption of 60 to 80 grams for the non-cirrhotic controls. In view of the long-held opinion that cirrhosis in alcoholics is merely the result of alcohol-induced dietary deficiencies, it was particularly noteworthy to see that neither calorie nor protein content in the diet of these two groups differed significantly.

The observation that the delta-type of alcoholism prevails in France with its exceptionally high cirrhosis mortality, leads to the assumption that besides time and dose a third factor may be of significance, namely the continuity of alcohol influence. Appreciable periods of abstinence, during which the outstanding capacity of the liver parenchyma for regeneration is allowed to exert its preventive influence, will probably decrease the risk of cirrhosis. However, only a few histological studies have attempted to compare the incidence of cirrhosis in groups of drinkers with continuous versus intermittent or periodic alcohol abuse. The results, although partly contradictory, seem to favour the opinion that unremitting, habitual over-indulgence is more dangerous than intermittent or periodic, even gargantuan drinking bouts (Chalmers, Murphy and Taft, 1948; Edmondson, Peters, Frankel and Borowski, 1967; Leevy and Smith, 1969; Wilkinson, Santamaria and Rankin, 1969; Viel, Salcedo, Donoso and Varela, 1970; Insunza, Iturriaga, Ugarte and Altschiller, 1971).

Being acquainted with these earlier results and the complexity of the problem, I had occasion to examine 526 male alcoholics from all social strata who were consecutively admitted to a special sanitarium in West Germany, for voluntary withdrawal treatment (Lelbach, 1966; 1967 a and b; 1968; 1972b). This investigation was started with the aim of collecting data on clinical, biochemical and histologic findings in a group large enough to permit, firstly, a statistical evaluation of the two essential variables (*i.e.* the duration of alcohol abuse and the intake per time unit) and then to assess their potential correlation to liver damage. It must be stressed that this institution did not admit patients who needed special medical treatment on admission or who presented symptoms of alcoholic psychosis. This eliminated any bias in favour of an over-representation of advanced liver disease in the sample. The patients included in this fairly homogeneous group were, in the majority, alcoholics for whom there was hope for re-adjustment to social life. Alcoholics in the end-stage of addiction and the skid row type were rare. This is borne out by the fact that for 86.5 per cent it was the first withdrawal treatment and that previously experienced episodes of alcoholic psychosis were reported by only 8.6 per cent of the total sample. The age ranged between 19 and 64 years with a mean of 40.0 ± 10.5 years and an average history of excessive alcohol consumption of 9.1 ± 5.8 years. Carefully collected anamnestic data as well as clinicobiochemical findings ruled out that previous liver disease of non-alcoholic origin or a severe protein malnutrition had played any demonstrable etiologic role in the final degree of liver damage diagnosed in the cases of this group.

In view of the difficulties to be encountered in obtaining data on drinking habits, all drinking histories were taken by myself according to a detailed questionnaire at the end of a first interview. Data on duration of alcohol abuse and mean intake per day or per week were re-checked for each patient by the psychiatrist in charge of the institution in a second separate interview which was carried out at a later date. In addition, all quantitative data contained in records of social agencies responsible for admission procedures, information furnished by family physicians and next of kin, as well as all available records on hospital treatment in the past were collected and processed. In view of the well-known tendency to minimize the actual intake, the final computation was based on the highest figures admitted for duration and dose per time unit. It was felt that this procedure might yield a body of data on which a statistical evaluation could be based in a fairly reliable manner.

As it turned out, figures on duration of heavy drinking were easier to obtain than figures on average quantities consumed. Only in 417 patients did records on average intake seem reliable enough to justify inclusion in the final calculation. Each patient was questioned about his average minimum and maximum intake per day and per week for the successive stages in his drinking life and the amounts reported were expressed in grams of absolute alcohol per day, so that, for instance, in week-end drinkers, drinking days were averaged with abstaining days. An average for all minimum and maximum quantities were computed, based upon the highest figures recorded during the individual drinking histories, irrespective of a final dose reduction due to symptoms of alcohol intolerance. It should be emphasized that data on drinking histories were collected and processed before results of biochemical tests and liver biopsies were known.

TABLE III

RANGE OF AVERAGE DAILY ALCOHOL CONSUMPTION
IN 417 ALCOHOLICS TREATED AT A SPECIAL SANITARIUM^a

	No. of Cases	Per Cent of Sample	Alcohol Intake in Grams Per Day		Mean Duration of alcohol Abuse (years)
			Mean	Average of minimum and maximum quantities consumed	
Reliable data on amounts consumed	417	100	178.5	145-212	9.1
Below 160g/day	186	45	124	99-149	7.4
Above 160g/day	231	55	224	182-266	10.3

^aLelbach, 1966, 1967a

As can be seen from Table III, the average minimum and maximum consumption ranged from 145 to 212 g of ethanol per day with a mean of approximately 180 g. Forty-five per cent of the sample had had a mean daily ethanol intake of less than 160 g, averaging 124 g per day; these were mostly younger alcoholics with a weekend drinking pattern, or alcoholics who only admitted periodic bouts. Patients with a mean daily intake exceeding 160 g were recruited almost exclusively from the class of steady daily

imbibers. In general, mean daily intake tended to rise concomitantly with increasing duration of abuse but within an individually widely varying range. Since alcoholics tend to minimize their consumption rather than to overestimate previous drinking habits, it can be assumed that actual intake, especially during bouts, was more likely to have exceeded the figures reported here. However, it was particularly interesting to know that figures on mean daily intake of alcoholics collected from samples of different nationalities for the specific purpose of determining average consumption in labelled alcoholics were surprisingly similar, with means lying between 175 and 220 g per day (Von Lutterotti, 1964; Vetter, 1966; Schmidt and Popham, 1968; Wilkinson, Santamaria, Rankin and Martin, 1969; Grünberger, Irsigler and Kryspin-Exner, 1970). In 1956, Ledermann had coined the phrase of the "terrain éthylique" meaning a range of daily consumption between 160 and 240 g, and this still seems to be a good average estimate which holds true throughout the world.

TABLE IV
INCIDENCE OF CIRRHOSIS IN LIVER BIOPSIES
OF 320 ALCOHOLICS AND OF A SMALLER SUB-GROUP^a

	No. of Cases	Per Cent of Sample	Duration of alcohol abuse in years (mean \pm 1SD)
Total Sample (N = 320)	39	12%	9.9 \pm 5.9
Subgroup (N = 157)			
Alcoholics with a daily intake exceeding 160g. (mean = 226g minimum-maximum: 186-268g)	39	25%	11.4 \pm 6.5

^aLelbach, 1966 and 1967a

Among the total sample of 526 patients, well-established cirrhosis of the liver was found clinically and/or histologically in 53 cases (10%). More correctly, and resulting in a slightly higher percentage, the overall prevalence of cirrhosis was reflected in a group of 320 patients in whom liver biopsy was performed; histology revealed cirrhosis in 39 cases (12%) (Table IV). These figures seem to fit with Klatskin's estimate (1961). However, as a selected sub-group shows, the incidence of cirrhosis was more than twice as high when daily alcohol consumption had exceeded 160 g of ethanol. From this Table, two conclusions can be drawn. 1. Cirrhosis was not found in those whose daily consumption had been less than 160 g. 2. After roughly twelve years of alcohol abuse at a level of intake exceeding 160 g per day histology revealed cirrhosis in 1 out of 4 alcoholics. Considering the last point, Wilens' autopsy series of 519 alcoholics (1947) with a daily consumption of one pint of whiskey or more should be remembered since the incidence of cirrhosis

TABLE V
INFLUENCE OF DURATION OF ALCOHOL ABUSE
ON THE INCIDENCE OF CIRRHOSIS
IN 334 ALCOHOLICS^a

Mean Duration of alcohol abuse in years (range)	No. of Cases	Cirrhosis in per cent of cases	Mean daily consumption in g. ethanol
3.6 (1-5)	73	0%	163 (130-197)
8.3 (6-10)	129	8%	177 (144-210)
12.9 (11-15)	81	21%	192 (160-224)
21.6 (16-35)	51	51%	227 (197-275)
Total 334			

^aLelbach, 1966 and 1967a

was quite similar in his sample (27.9%); in terms of g ethanol; one pint equals 160 g for 86-proof whiskey and 188 g for 100-proof whiskey.

As a next step, the cases were arranged according to the duration of alcohol abuse (Table V). They were allotted to four groups with a duration of (a) 1 to 5 years, (b) 6 to 10 years, (c) 11 to 15 years, and (d) more than 15 years. Arranging the cases in this manner made it quite clear that the relative frequency of cirrhosis in the sample did rise almost linearly with increasing duration of excessive alcohol use. Whereas no case of cirrhosis could be found among those who drank excessively for a period up to 5 years, the incidence of cirrhosis rose to finally 51 per cent of the cases when alcohol abuse had lasted on the average for almost 22 years. However, it also became evident that the influence of duration of heavy drinking was not an isolated factor, since parallel to increasing duration of abuse a definite rise in daily consumption could be observed. This was accompanied in most patients by switching over to the use of more concentrated beverages (Lelbach, 1967b, 1968).

It was next attempted to determine the differential influence of the two variables, dose and time, and especially to assess the effect of two different levels of daily intake independent of the duration of abuse. For this purpose, the histologic results were compared of two selected sub-groups comprising 108 cases each, which had been matched for age, duration of heavy drinking and body weight. The two groups differed only in respect to average daily intake which was 126 g of ethanol for group 1 and 226 g for group 2. As can be seen from Table VI, the number of cases with uncomplicated fatty liver were practically identical in both groups but the percentage of potentially precirrhotic lesions, *i.e.* the number of cases with steatosis plus fibrotic and inflammatory changes and with chronic alcoholic hepatitis, was more than twice as high in group 2. Whereas no case of cirrhosis could be found in group 1 with an intake of less than 160 g per day, cirrhosis morbidity was 14 per cent after an intake of more than 160 g for an average of approximately 8 years. It thus becomes evident that the average alcoholic can be expected to

suffer from cirrhosis of the liver — provided that other cirrhogenic factors were absent — only if a certain level of intake of alcoholic beverages per time unit is kept up for a certain minimum range of time.

TABLE VI
INFLUENCE OF DIFFERENT LEVELS OF CONSUMPTION
ON THE LIVER AS EVIDENCED BY COMPARING HISTOLOGY
OF TWO OTHERWISE CLOSELY MATCHED GROUPS OF ALCOHOLICS.

	Group 1 (n = 108) Less than 160g/day (100-152g; mean = 126.5g/day)	Group 2 (n = 108) More than 160g/day (188-266g; mean = 227g/day)
Duration of alcoholism in years ($\bar{x} \pm SD$)	7.9 \pm 4.1	7.9 \pm 3.8
Diagnosis:	HISTOLOGY: (in per cent of sample)	
normal histology:	40%	10%
moderate to severe fatty infiltration:	46%	43%
potentially precirrhotic lesions (severe steato- fibrosis with inflam- matory reactions: chronic alcoholic hepatitis)	14%	33%
CIRRHOSIS	0%	14% ¹

¹ 2 p < 0.0005

However, one could argue that any suspected correlation between levels of daily intake and resulting degree of liver damage cannot be correctly interpreted unless it is established on a body weight basis; otherwise an individual distribution factor for ethanol would be disregarded. A number of investigators have estimated that in the human body the rate of ethanol elimination ranges usually between 70 and 132 mg/kg/hour (Goldberg, 1943; Elbel and Schleyer, 1956; Lundsgaard, 1956; Liebermann, 1963; Barnes, Cooke, King and Passmore, 1965; Reynolds, Redeker and Kuzma, 1965; Trémoliers, Lowy and Griffaton, 1967; Greenberg, 1968). Thompson (1956) reported a wider range of 50 - 180 mg/kg/hour, the latter value representing an absolute maximum rate of disposal already accompanied by an elevation of blood alcohol level. If maximally possible daily consumption of alcohol is defined as the intake per time unit that does not entail an appreciable increase of blood alcohol levels while at the same time fully saturating the pathways of alcohol metabolism provided that the intake is evenly spaced, the range of about 100 to 130 mg/kg/hour, would still be a good average estimate (Greenberg, 1968), as originally

proposed by Widmark (1933). Basing his opinion on an extensive series of experiments, Goldberg (1963), who is certainly an expert in this field, again recently emphasized that the total amount of alcohol eliminated per hour is clearly correlated with body weight which means that a bigger person can generally get rid of higher amounts of alcohol per hour than a person weighing less. Goldberg quoted the example of an individual weighing 100 kg with an elimination rate of 125 mg/kg/hour which he expressly noted as a rather high rate. In order to meet the above mentioned argument, it was therefore thought necessary to calculate in addition, amounts of mean daily intake on a body weight basis. The results for different stages of alcoholic liver disease are shown in Table VII. Two conclusions can be drawn from this table: 1. Calculations of mean daily intake on a body weight basis showed proportionally the same correlation with increasing degrees of histologically diagnosed liver damage as prior calculations disregarding this ratio. 2. For potentially precirrhotic lesions and particularly for cases with cirrhosis of the liver, mean intake per kg of body weight had been more or less continuously either in the range of estimated maximum rate of elimination or above this upper limit. Furthermore, it must be borne in mind that in actual drinking practice, alcohol intake can at best be spaced over the 16 waking hours of the day whereas this calculation was based on a 24-hour day. Therefore, the true figures for intake per kg body weight and hour during drinking hours must have been at least 33 per cent higher.

TABLE VII
ALCOHOL INTAKE IN RELATION TO BODY WEIGHT
AND HISTOLOGIC DIAGNOSIS^{a, b}

Diagnosis	No. of Cases	Daily intake in mg/kg/hour		Duration of alcohol abuse in years (Range) Mean \pm SD	
		Mean	Average of Minimum-maximum		
Normal histology	70	90	69-112	(2-18)	7.7 \pm 4.1
Uncomplicated fatty liver	118	109	91-128	(1-38)	7.8 \pm 4.7
Severe steatofibrosis with inflammatory reactions	44	127	102-152	(2-30)	10.3 \pm 5.5
Chronic alcoholic hepatitis	48	125	105-145	(2-26)	11.9 \pm 5.2
Cirrhosis	39	147	118-175	(6-30)	17.1 \pm 6.8
Total	319				

^aLefbach, 1966 and 1967a

^bOn the basis of a 16-hour drinking day (= plus 33%) figures for *mean intake* during drinking hours would have been: 120, 145, 169, 167, and 186 mg/kg/hour, respectively.

All this strongly suggests that severe liver damage and particularly cirrhosis of the liver is likely to develop if an influx of alcohol per time unit that quantitatively exceeds the capacity of the mechanisms responsible for elimination is maintained for prolonged periods. This hypothesis, which makes allowance for the ambivalent nature of the ethanol molecule, would offer a plausible explanation for the divergence in opinion on whether alcohol is a hepatotoxic agent or not. Ethanol is to a certain extent fully metabolizable if the influx per time unit does not exceed an individually determined upper limit; beyond this limit which is indicated by a rise in blood alcohol level, however, it exerts a definitely toxic influence as is well known from its effect on the central nervous system. As soon as blood alcohol levels begin to rise, the ensuing toxic effect becomes dose-dependent.

Since only the combined influence of dose and time would yield a representative measure for studying any quantitative relation between alcohol abuse and resulting liver damage, a correct visualization of this interdependence would properly require a three-dimensional presentation with dosage and duration being the two variables. But as maximally possible alcohol consumption per day is self-limiting, total amounts of alcohol consumed by an alcoholic during his drinking life are largely dependent on the length of his drinking history, and thus become a function of time. It can therefore be rightly assumed that, on a body weight basis, a hypothetical product of mean daily intake in grams of ethanol and length of drinking history, *i.e.* g/kg/day multiplied by years, would yield a figure into which not only dosage and time of exposure enter equivalently but which also makes allowance for an individual distribution factor. By this procedure, the dilemma of a three-dimensional presentation could be legitimately circumvented. As a final step in the investigation of this complex interrelationship, it was therefore attempted to study whether any demonstrable correlation existed between the product of total amount of alcohol consumed over the years of excessive drinking and the incidence of (1) precirrhotic lesions and (2) cirrhosis of the liver.

For this purpose, a last group was composed consisting of all those alcoholics contained in the total sample for whom histologic results as well as presumably reliable data on duration of alcohol abuse and average daily intake were available. This group comprised 265 alcoholics. Of these, 239 had been steady daily drinkers, 24 had a "weekend drinking" pattern; only two had been "spree-drinkers" with drinking bouts which had been interrupted by short periods of abstinence. For each of these 265 patients the dose-by-time-product was computed.

The values obtained were then grouped according to rank into 13 classes. Within each class they were plotted against (a) the percentage of severe liver damage defined as exceeding in degree uncomplicated fatty liver (108 cases), and (b) the percentage of cirrhosis (39 cases). In view of the unavoidable shortcomings of a retrospective study of this kind and despite all endeavours to corroborate possibly unreliable data obtained by cross-checking with results from different sources of information, I was quite prepared to expect at best only a loose correlation.

It was therefore most surprising to find that an analysis of regression revealed an astonishingly high degree of correlation between alcoholism as expressed in terms of total amounts consumed during drinking life and the incidence of severe liver damage or cirrhosis of the liver, respectively (Figs. 1 to 3). The coefficient of correlation ($r = 0.95$ or 0.98 , respectively) corresponded to a probability of error of less than 0.001. This correlation resembles a dose-effect-relationship but this still leaves room for individual susceptibility. Péquignot (1958, 1961, 1963) had estimated that a dose of 180 g of ethanol per day consumed for roughly 25 years could be considered as an "average cirrhogenic dose"; this would correspond to a total intake of approximately 4,200 litres of 100-proof

whiskey or 21,000 litres of French wine (10% v/v) at a body weight of 70 kg. If one applies Péquignot's estimate to Figure 2 it can be seen that for the group of German alcoholics presented here, cirrhosis would be expected in only every second alcoholic after a total dose of this magnitude. Or, if one assumes that a primarily healthy subject weighing 70 kg would be capable of maintaining a daily intake of about 210 g of ethanol, for about 20 years, which corresponds roughly to 21 oz. of 86-proof whiskey per day, he would have a 50 per cent chance of suffering from cirrhosis. This interpretation also makes it clear that individual susceptibility seems to be of equal importance to dose and duration of alcohol abuse. However, it does not give any clue as to how this still somewhat mysterious concept of susceptibility is to be defined. It can be assumed — and this is hypothetical — that susceptibility in the sense of an "Anlage-factor" will be related to the inborn capacity of alcohol metabolism and the individual's ability to react with an enzyme-induction or an enhanced reoxidation of NADH_2 . Another aspect of susceptibility is the maintenance of pre-existing capacity for metabolizing ethanol. It was recently shown (Bode, Buchwald and Goebell, 1971) that a short period of severe protein malnutrition very effectively reduces the capacity of mechanisms responsible for alcohol meta-

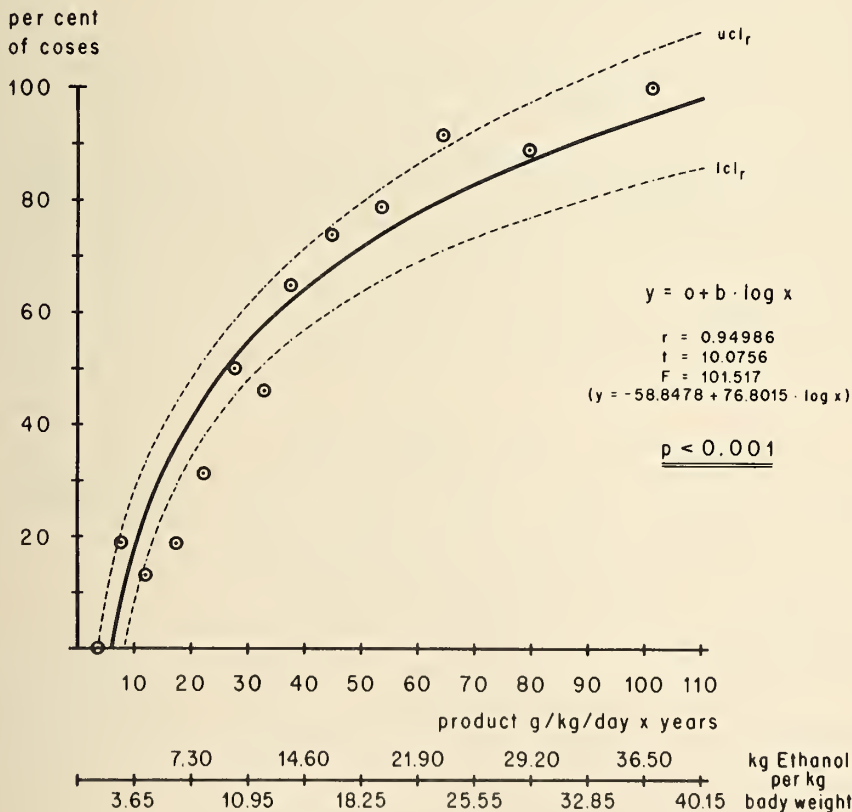


Figure 1. Correlation between total amount of ethanol per kg of body weight consumed during drinking life and incidence of severe liver damage ($n = 108$; severe steatofibrosis with inflammatory reactions, chronic alcoholic hepatitis, and cirrhosis of the liver) in 265 alcoholics (Lelbach, 1972. Reproduced by permission of the publisher, Georg Thieme Verlag, Stuttgart.)

— ucl_r; lcl_r = upper and lower confidence limits of regression

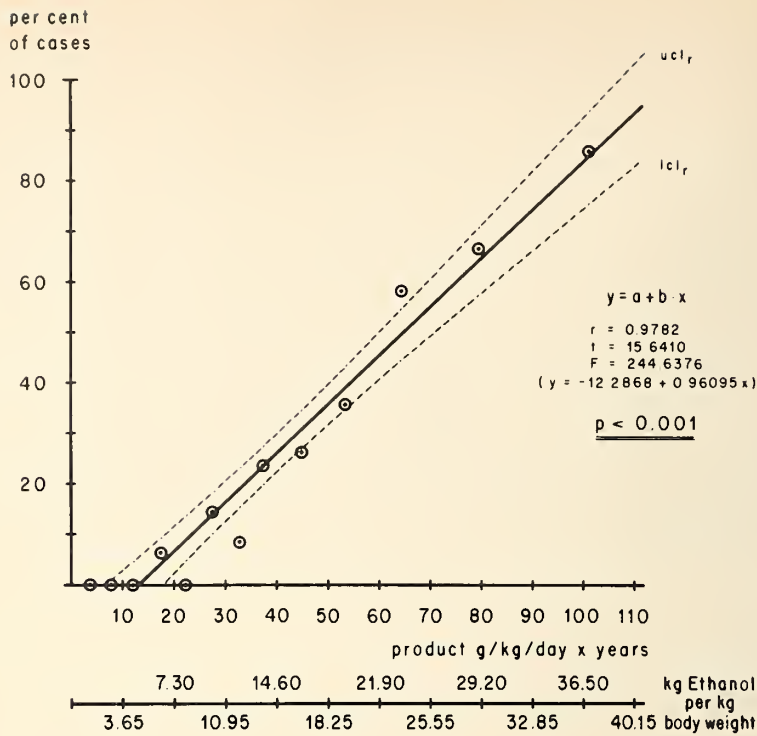


Figure 2. Correlation between total amount of ethanol per kg of body weight consumed during drinking life and incidence of cirrhosis of the liver (n = 39 cases) in 265 alcoholics (Lelbach, 1972. Reproduced by permission of the publisher, Georg Thieme Verlag, Stuttgart.)

— ucl_r; lcl_r = upper and lower confidence limits of regression

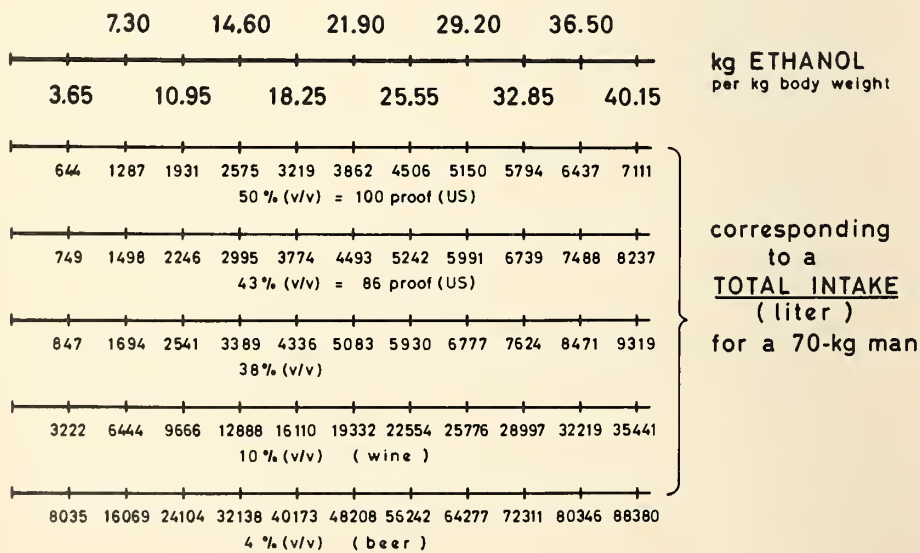


Figure 3. Total amounts of ethanol per kg of body weight expressed as liter per 70 kg body weight for various alcoholic beverages (Lelbach, 1972. Reproduced by permission of the publisher, Georg Thieme Verlag, Stuttgart.)

bolism. I am quite convinced that the relationship between dietary deficiencies and liver disease in the alcoholic, which in the past has been emphasized (Rubin & Lieber, 1968), is in reality only a phenomenon of secondary importance insofar as a protein deficiency impairs the mechanisms of alcohol degradation and the regenerative capacity of hepatic parenchyma.

As a warning against misinterpretation, however, I would like to put emphasis on three points: 1. Any additional enhancing factor, such as liver disease of non-alcoholic etiology and/or nutritional imbalance will increase the risk of cirrhosis in the alcoholic to an unpredictable extent. 2. Dose and duration are not really interchangeable. 3. In view of the wide individual variations in alcohol metabolism and in the susceptibility to liver damage and also in view of other as yet non-definable factors, it would most certainly be unwarranted to draw any predictive or retrospective conclusions *for an individual case* from these collective data, let alone to construe some kind of a "formula".

It has often been proposed that not ethanol but other ingredients in alcoholic beverages, (especially various congeneric substances), might be responsible for liver damage in alcoholics. For the study of this problem two groups of beer and spirit drinkers were compared (Lelbach, 1967b & 1968). Almost half of the alcoholics of the total sample drank only beer (259 patients) but exclusive use of spirits was reported by not more than one-tenth of the cases (52 patients). These 52 spirits drinkers were matched with 52 beer drinkers as to duration of heavy drinking and daily quantities consumed, as well as to age, body weight, length of abstinence period, and previous illnesses (Tables VIII and IX). Neither the assessment of functional liver damage nor histologic results revealed a significant difference between the two groups, indicating that the degree of liver damage obviously depended on the total amounts of alcohol consumed and not on the type of beverage.

The relationship between alcohol abuse and cirrhosis of the liver demonstrated in this study requires one interpretative comment. It is true that cirrhosis was found in one out of four alcoholics after a daily intake of about 230 grams for roughly 12 years and in

TABLE VIII

RELEVANT PARAMETERS OF TWO MATCHED GROUPS OF
BEER AND SPIRIT DRINKERS^a

	Biochem. Results		Histology	
	Beer Drinkers	Spirit Drinkers	Beer Drinkers	Spirit Drinkers
No. of cases	52	52	40	35
Duration of abuse in years ($\bar{x} \pm$ SD)	11.9 \pm 6.9	11.9 \pm 7.6	13.3 \pm 7.0	13.8 \pm 7.9
Average daily intake in g. ethanol (minimum-maximum)	209.5 (171-248)	209.5 (170-249)	214 (175-253)	224 (179-269)
Age in years ($\bar{x} \pm$ SD)	40.8 \pm 10.6	44.5 \pm 10.3	42.2 \pm 10.6	45.6 \pm 8.8

^aLelbach, 1967b and 1968

TABLE IX
COMPARISON OF BIOCHEMICAL RESULTS AND
HISTOLOGY IN TWO MATCHED GROUPS OF
BEER AND SPIRIT DRINKERS^a

	Beer Drinkers (n = 52)	Spirit Drinkers (n = 52)
Biochemical Results		
Functional liver damage semi-quantitatively graded †† and ††† ^b	22 cases (42%)	22 cases (42%)
	(n = 40)	(n = 35)
Histology		
Liver damage exceeding uncomplicated fatty liver	23 cases (58%)	22 cases (63%)
Cirrhosis	13 cases (33%)	11 cases (31%)

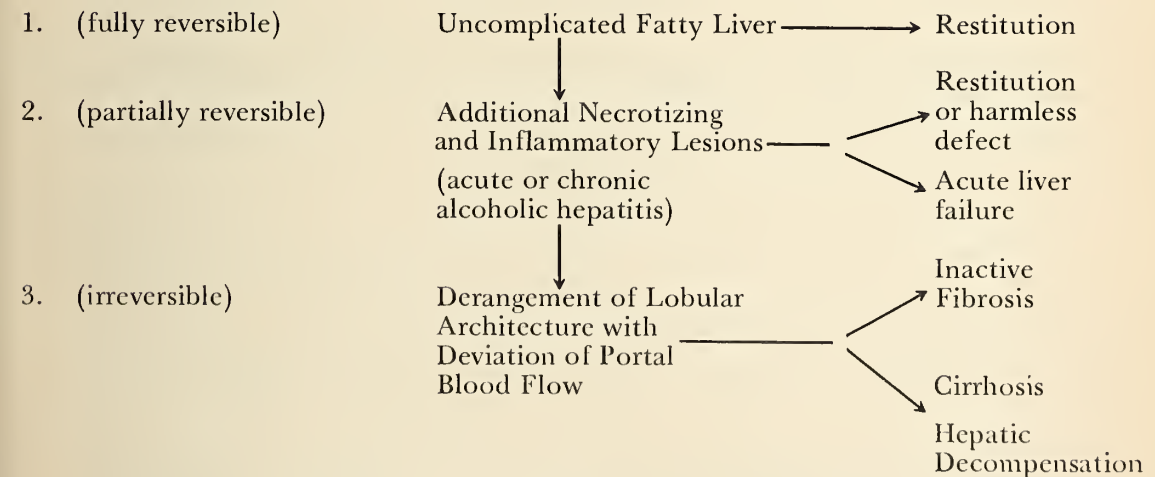
^a Lelbach, 1967b and 1968
^b = 2 or more of a battery of tests at least moderately pathologic and one of them highly abnormal (the battery included: sulfobromophthalein test, serum total bilirubin, glutamic pyruvic transaminase, glutamic oxalacetic transaminase, alkaline phosphatase, cholinesterase, urinary urobilinogen, thymol turbidity, Takata-Ara reaction, total protein, albumin, gamma-globulin.)

every second drinker after the same dose ingested for 22 years. This, however, should not be interpreted as indicating that the development of cirrhosis in the alcoholic is a continuous process which takes 10 or 20 or 25 years. Rather, irreversible liver damage is usually initiated rather suddenly at some point along the time axis by a degenerative, necrobiotic process the exact nature of which is as yet not fully understood. It is a phenomenon well known to clinicians and pathologists alike that a minority of alcoholics may continue to ingest considerable amounts of alcohol well up to old age without exhibiting more than simple fatty liver at autopsy. But in the majority, alcohol-induced fatty liver which can persist for many years and still remain completely reversible by abstinence alone will be complicated some day by necrobiotic lesions which evoke inflammatory and fibrogenic reactions as well as the regenerative power of the hepatic parenchyma (Table X). As Thaler (1969) and Denk, Leodalter, Schnack and Thaler (1970) demonstrated by repeat biopsies, the development of this next and decisive stage of alcohol-induced liver disease can take place within a relatively short period lasting from several months to a few years. This intermediate, transitional stage is usually encountered after not less than 5 years and on the average after 10 to 13 years of heavy drinking (Lelbach, 1966; 1967a); it can be regarded as a potentially precirrhotic process and is known as acute or chronic alcoholic hepatitis. Alcoholic hepatitis is still at least partially reversible depending on the successful elimination of the noxious agent and on the regenerative capacity of hepatic parenchyma.

The acute alcoholic hepatitis usually becomes manifest after a prolonged period of ingestion of particularly large quantities of alcohol; it can rapidly terminate in acute liver failure but may also quickly progress to an active form of cirrhosis. Probably more dangerous, however, is the chronic form of alcoholic hepatitis because of its insidious onset and its gradual progression to a derangement of hepatic lobular architecture with finally increasing obstruction of portal blood flow. If these lesions respond favorably to therapy (strict abstinence and a nutritious diet) they may finally be brought back to an inactive fibrosis which will do little if any harm. More likely, however, is a progression to frank micro-nodular cirrhosis within months and years. It should be realized that in this respect not only the labelled alcoholic is at risk but to the same degree so is the regular social drinker who ingests similarly large quantities but who has learned to space his drinks evenly over the day so that overt inebriation and gross impairment of social function are avoided.

Once hepatic architecture is disturbed to a degree that hepatic circulation of portal blood is seriously impeded, the process becomes more and more autonomous. This makes it understandable that the favourable influence of abstinence on survival seen in mild and moderately advanced alcoholic cirrhosis as reported by Powell and Klatzkin (1968) and by Rankin *et al.* (1970) could not be demonstrated for cases with advanced portal hypertension. (Jackson, Perrin, Smith, Dagradi and Nadal, 1968; Soterakis, Resnick and Iber, 1973).

TABLE X
DEVELOPMENTAL STAGES OF
ALCOHOL-INDUCED LIVER DISEASE^a



^aLelbach, 1972

SUMMARY

The attempt was made to analyze the complex interrelation of a number of quantitative factors of alcohol abuse and their importance in the development of alcoholic cirrhosis in man. The results derived from studying the incidence of liver damage among a group of 526 institutionalized alcoholics have shown that a direct correlation exists between the

intensity of alcohol influence and the degree of liver damage. At the same time, it was made clear that the concept of intensity of alcohol influence is at least a two-dimensional problem in which dose and time of exposure and also its continuity play the dominant role. This may help to clarify and dissolve the apparent discrepancy between an evidently high incidence of alcoholism in the etiology of cirrhosis of the liver throughout the world and the seemingly low percentage of cirrhosis among alcoholics, as a merely cross-sectional type of investigation is likely to suggest. In reality, the risk an alcoholic incurs of suffering from cirrhosis of the liver depends primarily on whether he is in a position to drink long enough and hard enough; a definition of what is "hard enough", meaning dose per time unit, depends on the individually varying capability to dispose of alcohol. With a view to the nutritional implication of a high alcohol intake, Hartroft (1967) who doubts a hepatotoxic action of ethanol had rightly asked: "What are the dose-response curves for alcohol if it is a toxin?" It seems that, indeed, some kind of dose-effect relationship exists, but I also hope to have made it clear that this relationship is a complex one. As opposed to other hepatotoxic agents, the ethanol molecule has an ambivalent nature. Up to a certain influx per time unit it is a substance the metabolism of which yields fully utilizable calories; within this range the influence of alcohol remains a metabolic problem to be dealt with by the different metabolic pathways. Beyond this limit, however, its toxic nature becomes evident, and this limit will be lowered if alcohol abuse entails a nutritional deficiency, especially a protein malnutrition. I am quite convinced that it is this double-faced nature of alcohol which has made it so difficult to prove a sort of dose-response relationship.

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Agreement, Disagreement in Experimental, Clinical and Epidemiological Evidence on the Etiology of Alcoholic Liver Cirrhosis: A Comment

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For present purposes, these three methods of investigation are delineated as follows: “experimental” refers to studies of the effect of alcohol on the liver in animals; “clinical” refers to studies in which prevalence and severity of liver pathology in clinic populations is related to drinking histories; and “epidemiological” refers to the study of spatial and temporal variation in liver cirrhosis mortality. The following potential etiological factors will be considered:

1. Volume and duration of alcohol use.
2. Drinking pattern (continuous vs. periodic drinking).
3. Type of alcoholic beverage.
4. Nutritional deficiencies.
5. Urbanism.

VOLUME AND DURATION OF ALCOHOL USE

Volume of Consumption

Clinical investigations have shown that the risk of developing alcoholic cirrhosis increases with the quantity of alcohol consumed (Lelbach, 1974). Drinking histories of alcoholics usually indicated that the level of consumption increases with the number of years of alcohol abuse (de Lint, Popham and Schmidt, 1970). For this reason, it is usually not possible to determine the effect of quantity independent of duration of drinking, and vice versa. At least one investigator was able to compare groups of alcoholics with similar duration of heavy alcohol use but different quantities of intake and found a marked difference in the prevalence of liver cirrhosis (Lelbach 1967 a, b; 1972). On the basis of

this finding, he suggested that average intake and duration of abuse are additive factors and that a direct relationship exists between the amount of alcohol consumed over a drinking life and the final degree of liver damage.

As may be expected, the alcohol consumption of drinkers en masse is also reflected in the mortality from liver cirrhosis in general populations. The most common way of demonstrating this association consists of correlating per capita consumption of alcoholic beverages and rates of death from this disease. Evidently these two variables are not fully satisfactory for this purpose: the mortality data include cases of cirrhosis unrelated to alcohol use and the per capita consumption is based on the total volume of alcohol use in the general population. We have to assume, therefore, that (1) the rate of death from cirrhosis that is unrelated to alcohol use is similar among the populations in the comparison, (2) the proportion of drinkers of cirrhogenic quantities rises and falls with the level of overall consumption. The first assumption is tenable in limited temporal comparisons and also in regional comparisons of relatively homogenous populations (Jolliffe and Jellinek, 1942). Concerning the second assumption, data from several populations have indicated that the distribution of alcohol consumption is one parametric and of the logarithmic normal family (Ledermann, 1954). This implies that an increase in the per capita consumption of alcohol is always accompanied by an increase in the proportion of heavy consumers, no matter what level of consumption is used to distinguish heavy from other use. One would expect, therefore, that the utilization of vital and consumption statistics should bear out the inferences drawn from clinical observations. To indicate the degree of relationship between per capita consumption and liver cirrhosis mortality, the results of some temporal and regional correlation-analysis are shown in Table I.

TABLE I

TEMPORAL AND REGIONAL CORRELATIONS BETWEEN RATE OF LIVER
CIRRHOSIS MORTALITY AND PER CAPITA ALCOHOL CONSUMPTION

Based on Popham (1970)

Area	Series	Correlation Coefficient	Probability
Australia	1938-59	.65	< .005
Belgium	1929-59 (less 1940-5)	.75	< .001
Canada	1927-60	.88	< .001
Alberta	1929-60	.85	< .001
Manitoba	1935-60	.86	< .001
Nova Scotia	1932-60	.60	< .001
Ontario	1930-60	.89	< .001
Quebec	1929-60	.43	< .05
Saskatchewan	1943-60	.77	< .001
Canada	9 provinces 1955	.81	< .01
Finland	1933-57	.78	< .001
France	1925-58	.62	< .001
France	23 départements 1950	.76	< .001
Holland	1927-58	.57	< .001
Sweden	1926-56	.45	< .05
United States	1934-58	.60	< .005
United States	45 states 1939	.61	< .001
United States	48 states 1944	.78	< .001
United States	46 states 1950	.76	< .001
United States	46 states 1957	.86	< .001

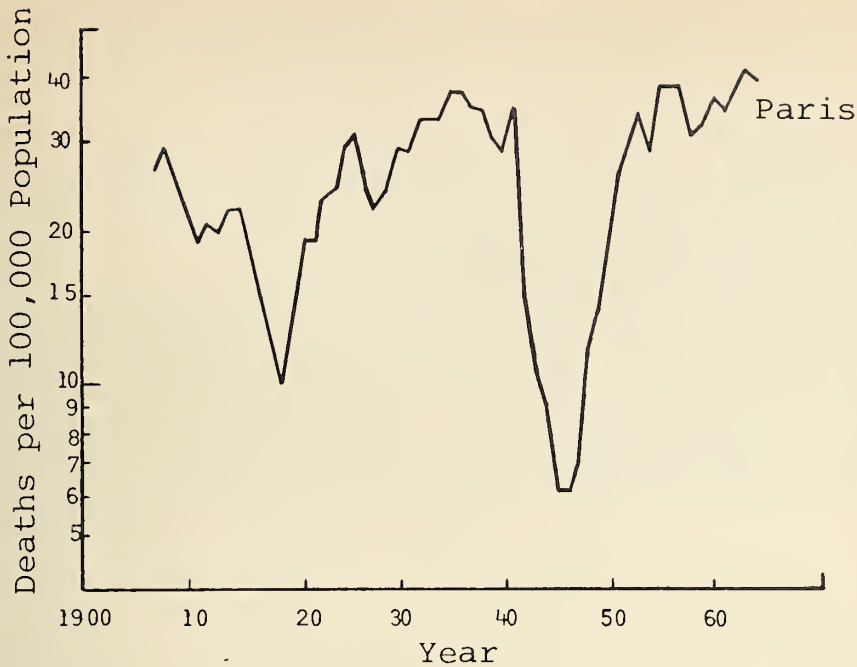


Figure 1. *Death Rates, Cirrhosis of the Liver, Paris, 1900-1964 Based on Ledermann (13).*

Temporal series of the type shown here are usually considered “weak design methods” that do not permit distinguishing spurious correlations. It is generally accepted that abrupt and decisive changes provide a better opportunity to test such associations than the gradual changes occurring over longer periods of time (Campbell, 1968; Wadman, 1971). French data for the years 1942-1945 provide us with an example of drastic and rapid reduction in per capita consumption over a relatively short period. The concurrent marked reduction in liver cirrhosis mortality (see Table II and Figure 1) constitutes strong evidence for an intimate association between the two variables.

TABLE II

REPORTED DEATHS FROM CIRRHOSIS OF THE LIVER PER 100,000 MALES
BY AGE IN FRANCE AND IN THE REGIONS BRETAGNE-NORMANDIE
(1935-1936 and 1942-1943)

Based on Ledermann (1964)

Age	France			Bretagne-Normandie		
	1935-36	1942-43	% Difference	1935-36	1942-43	% Difference
Males Age 35-49	46	20	-57	57	29	-49
Males Age 50-64	137	73	-47	153	84	-45

It has been suggested that the immediacy of the decline in cirrhosis mortality after the drastic reduction in the availability of wine and other alcoholic beverages is inconsistent with the clinical course of this disease which is marked by relatively long periods of heavy alcohol use. However, it is also known that the cirrhotic process can be halted or retarded by abstinence. This phenomenon probably explains the sharp decline immediately after the temporary reduction in consumption (Terris, 1967). Conversely, the rapid rise in these rates when alcohol again became freely available may be explained by further decompensation of previously established liver pathology.

It is important to note that the principal function of these correlations is not to add support to the clinical and experimental findings which have established the association between cirrhosis of the liver and alcohol use beyond dispute; rather it is to demonstrate that the mortality rates from this cause in general populations rise and fall with the overall level of alcohol consumption or, put differently, that the proportion of drinkers who consume quantities associated with the risk of alcoholic liver disease is directly related to the per capita consumption of alcoholic beverages in society at large.

Duration of Consumption

The importance of duration of abuse is reflected in the temporal series in Table III which shows coefficients of correlation between liver cirrhosis mortality for the years 1956-1965 and alcohol consumption data for a period preceding the former by five years (1951-1960). Evidently, the correlations obtained are very high and exceed those in the temporal series shown in Table I in which mortality and consumption refer to the same years. By this method, improvement in r values can usually be achieved when increases in consumption over time are more gradual than in the case of the French rates during World War II.

TABLE III

TEMPORAL CORRELATIONS (PRODUCT MOMENT CORRELATION COEFFICIENTS)
BETWEEN ALCOHOL CONSUMPTION IN THE YEARS 1951-1960 AND LIVER
CIRRHOIS MORTALITY IN THE YEARS 1956-1965 FOR VARIOUS COUNTRIES

Based on Wallgren, Kusunen and Nikander (1970)

Country	Spirits	Wine	Beer	Total
Belgium	1.0	0.95	0.99	0.99
Denmark	0.98	0.86	1.0	0.99
England	0.93	0.96	0.99	0.99
Finland	1.0	0.96	1.0	1.0
France	0.99	1.0	0.99	0.99
Germany	1.0	0.97	0.99	0.99
Holland	1.0	0.94	0.94	0.99
Hungary	0.97	0.99	0.94	0.99
Italy	0.99	0.97	0.98	1.0
Norway	0.99	1.0	0.99	0.99
Poland	0.94	0.95	0.98	0.96
Sweden	0.96	0.89	0.95	0.96
Switzerland	0.92	0.99	0.96	0.98
U.S.A.	1.0	1.0	1.0	1.0

*Magnitude of Change in Liver Cirrhosis Mortality
Relative to Change in Per Capita Consumption*

Coefficients of correlation are measures of association that do not reflect the magnitude of change in the dependent variable relative to a given change in the independent variable. For example, an r value does not tell us how large an increase in liver cirrhosis mortality could be expected if per capita consumption doubles. Estimates of such expected increases can be made on the basis of the known relationship between per capita consumption and the proportion of heavy drinkers in general populations. Thus, it has been observed that this relationship is parabolic, *i.e.*, increases in the proportion of heavy consumers will, on the average, be proportional to the square of the increase in per capita consumption (Ledermann, 1954; Skog, 1971). Accordingly, if the per capita consumption is doubled, we would expect a 300 per cent increase in the proportion of consumers of cirrhogenic quantities and eventually an increase of similar magnitude in the mortality from cirrhosis attributable to alcohol abuse.

When this assumption was tested, close agreement was found between increases in reported cirrhosis mortality through time and the increases expected on the basis of the postulated quadratic relationship between increases in consumption averages and rates of death from this cause. The relevant data and calculations are shown below.

Assuming quadratic relationship between increases in per capita consumption and increases in the rate of death from liver cirrhosis attributable to alcohol use and using the notation:

- C_1 = the per capita consumption of alcohol in year 1
- C_x = the per capita consumption of alcohol in year x
- D_1 = the rate of death from liver cirrhosis in year 1
- P_1 = the proportion of all deaths from liver cirrhosis which is attributable to alcohol use in year 1
- P_x = the proportion of all deaths from liver cirrhosis which is attributable to alcohol use in year x

then the assumption of a quadratic relationship is represented in equation 1:

$$\frac{P_x}{P_1} = \left(\frac{C_x}{C_1} \right)^2 \quad (1)$$

D_x (the rate of death from liver cirrhosis in year x) can be estimated by equation 2:

$$D_x = P_x D_1 + (D_1 - P_1 D_1) \quad (2)$$

where the first and last terms represent the proportions of death related and unrelated to alcohol use respectively. Substituting for P_x from equation 1 into equation 2:

$$D_x = \left(\frac{C_x}{C_1} \right)^2 P_1 D_1 + D_1 - P_1 D_1$$

Employing the data given in the Table below in this formula, expected rates from this cause of 13.0 (Canada, 1971) and 5.1 (Finland, 1968) are obtained. These values are very similar to the reported rates of 12.1 (Statistics Canada, 1973) and 5.4 (Official Statistics of Finland, 1969) respectively. This close agreement lends support to the assumption that the relationship between consumption averages and alcohol related cirrhosis death rates is quadratic.

	P_1 ¹	C_1	C_2	D_1 ⁴
Canada	.30 (1930)	3.9 (1930) ²	9.1 (1971) ³	5.6 (1930)
Finland	.38 (1954)	4.3 (1954) ²	4.6 (1968) ³	4.8 (1954)

¹While consumption and mortality data are routinely reported for many countries, P values can only be obtained through special studies. For Canada and Finland the values have been established through an analysis of temporal variation in liver cirrhosis mortality and through studies of drinking histories of persons who have died from this cause (Popham, 1956; Bruun, Koura, Popham and Seeley, 1960).

²The year taken as year 1 has to correspond to the year for which the P values have been established (Popham, 1956; Popham and Schmidt, 1958).

³The years 1971 (Canada) and 1968 (Finland) are the latest years for which these statistics were readily available (Schiedam, 1969; Statistics Canada, 1972).

⁴Rates of death per 100,000 population 20 years and older (Popham and Schmidt, 1958; Official Statistics of Finland, 1969).

CONTINUOUS VERSUS PERIODIC DRINKING

Experimental evidence indicates that, in pair-fed rats on marginal diets, the same amount of alcohol resulted in more severe liver damage when given continuously as dilute solutions than when given as spaced intoxicating doses (Wallgren, Ahlquist, Åhman, and Soumalainen, 1967). Clinical evidence appears to favour the assumption that continuous intake of alcohol is more likely to result in chronic liver pathology than the periodic consumption of very large quantities interspersed with abstinence or moderate alcohol use (Lelbach, 1974). However, since steady heavy drinkers usually consume larger quantities of alcohol over a drinking life than periodic drinkers, it is as yet not clear whether it is the total volume consumed over time or the spacing factor that is decisive. To answer this question would require the comparison of liver pathology in groups of heavy drinkers with identical intake over a drinking life but different spacing of consumption. Lelbach's work (1972) on the effect of dose and duration on frequency and severity of liver damage would suggest that volume over time rather than periodicity is of importance. While he found least damage in periodic and weekend drinkers, he also found that these drinkers ranked lowest in his volume-duration scale.

In comparisons of groups of alcoholic patients who evidently differed greatly in their respective drinking patterns — one containing many bender drinkers, the other many inveterate continuous drinkers — no difference was found in the rates of clinically diagnosable liver pathology (Schmidt, Smart and Moss, 1968). This finding was confirmed in a follow-up in which similar rates of death from liver cirrhosis were found in both groups (Schmidt and deLint, 1970). These findings led the authors to speculate that liver disease due to alcoholism is related to the total quantity of alcohol consumed over a drinking life, rather than to specific drinking patterns.

Evidence on the epidemiological level of inquiry appears to favour the hypothesis that continuous consumption is most likely to lead to chronic liver damage. In the western world, the viticultural countries — France, Italy, Spain, Portugal — report the highest rates of death from liver cirrhosis, while Scandinavian countries, particularly Finland and Norway, report the lowest rates (deLint and Schmidt, 1970). In the former, daily alcohol use integrated with everyday activities is the most common pattern, while in the latter, drinking occasions are typically infrequent but often result in a state of intoxication (Solms, 1962, 1964; deLint and Schmidt, 1970; Moeschlin and Righetti, 1970). But it must be kept in mind that these two groups of countries also differ vastly in the overall volume of alcohol consumed (deLint and Schmidt, 1970)

TYPE OF ALCOHOLIC BEVERAGE

Alcoholic beverages differ in the concentration of ethyl alcohol, the content of congeneric substances and other ingredients. In a recent review (Lelbach, 1974), it has been concluded that, on the clinical level of investigation, very little evidence has been forthcoming to support the hypothesis that these factors are responsible for liver pathology in humans.

Evidence from epidemiological investigations is less clear. For instance, in two separate investigations employing spatial comparisons of per capita consumption and liver cirrhosis mortality statistics it was found that the consumption of distilled spirits correlates more closely with rates of death from this disease than the consumption of beer or wine (Wallgren, 1960; Böttig, 1964). However, these studies have been questioned on several grounds, particularly the failure to control for cross-cultural differences in the proportion of drinkers and abstainers. In a regional comparison of the States of the Union, per capita wine consumption was found to be significantly more closely related to variation in liver cirrhosis mortality than the per capita consumption of beer and distilled spirits (Schmidt and Bronetto, 1962). (See Figure 2.) However, the authors' interpretation of this finding was that a larger proportion of wine than of beer or distilled spirits is consumed by excessive drinkers, probably because of its low cost. In the United States, some types of domestic wine constitute the least expensive source of alcohol of all legally sold beverages (Rubington, 1968; Docter, 1967). Thus, it was not concluded from these data that wine consumption compared to the consumption of beer or spirits leads more often to the development of cirrhosis of the liver.

There exists, then, no valid epidemiological or clinical evidence which would suggest that a certain amount of absolute alcohol consumed in one type of beverage is more likely to produce cirrhosis than when consumed in another type.

NUTRITION AND ALCOHOLIC LIVER CIRRHOSIS

Clinical evidence concerning the role of nutrition consists of two types:

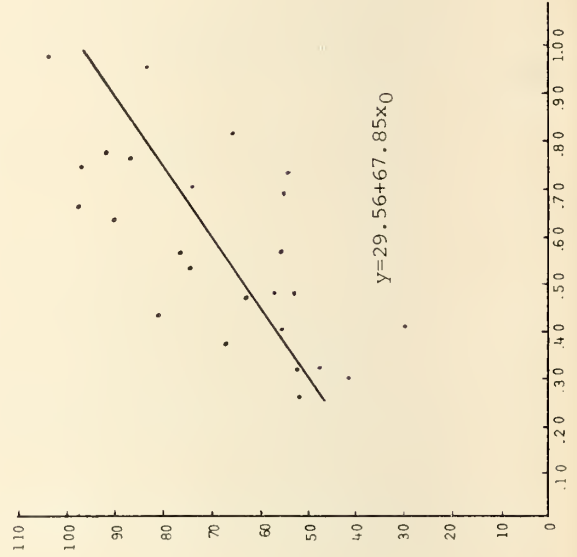
1. Body weight in samples of alcoholics is related to the frequency and severity of liver pathology. The bulk of evidence in these studies failed to show conclusively that this quantitative measure of dietary deficiency has any significant influence on the incidence and severity of liver pathology (Lelbach, 1974).



(a) The Regression of Liver Cirrhosis Mortality on Beer Consumption in the Eastern United States — 1950.



(b) The Regression of Liver Cirrhosis Mortality on Wine Consumption in the Eastern United States — 1950.



(c) The Regression of Liver Cirrhosis Mortality on Spirits Consumption for the Eastern United States — 1950.

Figure 2. Based on Schmidt and Bronetto (28)

2. In the second type, detailed histories of dietary habits of cirrhotics and controls are compared to determine whether the former had eaten a nutritionally less balanced diet (Péquignot, 1958). It was found that the two groups did not differ in the protein and caloric content of the diets.

To the best of my knowledge, no epidemiological data exist that have direct bearing on the role of nutrition in alcoholic liver disease. The periods of extreme food shortage that have occurred in Western countries were always accompanied by a very low consumption of beverage alcohol. It is, therefore, not possible to examine the effect of nutritional deprivation in general populations on alcohol related liver cirrhosis mortality. However, it is of interest to note that the earlier mentioned reduction in liver cirrhosis mortality in Paris during the latter years of World War II (Ledermann, 1964) occurred under conditions marked by severe food shortages.

Similarly, Germany and Austria reported very low rates of death from this cause in the early post war years (WHO, 1950), a period when famine-like conditions prevailed among large segments of society. Evidently, in general populations, reductions in cirrhosis mortality do occur in the presence of adverse nutritional conditions. Conversely, cirrhosis mortality in these jurisdictions rose rapidly in subsequent years when food supplies returned to normal.

Also of interest is the very high rate of death from liver cirrhosis reported from Portugal (WHO, 1972). In many parts of this country wine constitutes a less expensive source of calories than bread or other staple foods. As a consequence, heavy wine consumption often goes hand in hand with insufficient nutrition, particularly among the poorer segments of society (Bättig, 1967). Similar conditions have also been observed in some regions of South America, particularly among the Quechua in Peru and Bolivia (Popham, Notes on Peru). The study of the prevalence and nature of liver disease in these populations may shed some light on the role of nutrition in the etiology of alcoholic liver cirrhosis.

URBAN-RURAL DIFFERENCES IN LIVER CIRRHOSIS MORTALITY

In North America, liver cirrhosis mortality rates rise and fall with the extent of urbanism (deLint and Schmidt, 1970). It has been suggested that these differences can be explained by the higher levels of alcohol consumption in urban areas (deLint and Schmidt, 1970). However, available data do not always support this assumption (Schmidt and Bronetto, 1962). In Table IV are listed partial correlations between liver cirrhosis mortality rates, per capita consumption of wine, beer and spirits and degree of urbanism. The encircled coefficients of partial correlation in this Table indicate that, in the Eastern States of the Union, the relationship between liver cirrhosis mortality and urbanism remains close when the influence of per capita alcohol consumption is statistically controlled. One can, therefore, not reject the possibility that the urban environment per se is a significant determinant of variation in liver cirrhosis mortality.

The measure of urbanism in these correlations refers to the proportion of people living in cities of 50 thousand or over in each State and is thus a measure of population density. While the effect of this factor on physical pathology in humans is not known, some observations from animal ecology may be relevant in this context. It has been reported that the cycle of death rates of the Norwegian lemming and the Minnesota snowshoe hare depends on the rate of animals in a given space and not on the food supply as previously assumed. Apparently, increasing densities of the same species heighten

TABLE IV

PARTIAL CORRELATIONS BETWEEN LIVER CIRRHOSIS MORTALITY RATES (χ_4)
PER CAPITA CONSUMPTION OF WINE (χ_1), BEER (χ_2) AND SPIRITS (χ_0),
AND DEGREE OF URBANISM (χ_5) IN THE EASTERN UNITED STATES.

Based on Schmidt and Bronetto (1962)

	Eastern United States
* $r_{41.0}$	0.82
$r_{41.2}$	0.80
$r_{41.5}$	0.75
$r_{45.0}$	0.79
$r_{45.2}$	0.69
$r_{45.1}$	0.57
$r_{40.1}$	-.05
$r_{40.2}$	0.46
$r_{40.5}$	0.52
$r_{42.0}$	0.52
$r_{42.1}$	0.10
$r_{42.5}$	0.23

* r equals coefficient of partial correlation.

mutual stimulation beyond a critical point which triggers the compulsive flight preceding death. Autopsies revealed no signs of starvation but pathological changes in the adrenal cortex, hypoglycemia and liver cirrhosis or other hepatic damage. It has been suggested that these pathological changes resemble closely the stress or shock disease syndrome.¹ There seems to be experimental evidence in the same direction. Recently it has been shown that, in rats, stressful stimuli produce hepatic changes that are similar to those produced by ethanol administration (Israel, personal communication).

Evidently it cannot be argued that the variable urbanism corresponds to density in animal ecology or stress situations in experimentation. However, the evidence from these diverse areas of investigation suggest that a stress factor may account for some of the otherwise unaccounted-for differences in urban-rural liver cirrhosis mortality rates.

¹These notions stem from a paper on the Ecology of Alcoholism by J. R. Seeley (1967).

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Epidemiology of Alcoholic Liver Disease — Insights and Problems

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ALCOHOLIC LIVER DISEASE — AN INCREASING PROBLEM

An epidemiological approach is taken in this paper to some of the facts, problems and questions concerning alcoholic liver disease, in order to provide some clues and insights into its possible aetiology, prevention and treatment.

The prevalence of alcoholic liver disease and its consequent morbidity and mortality has, with some minor fluctuations, been progressively increasing in Canada since 1921. (Secley, 1960; Popham, Schmidt, and de Lint, in press). The death rate from liver cirrhosis per 100,000 adults rose from 2.9 in 1921 to 12.4 in 1968 (A.R.F. 1957-71), and during this period has accounted for a progressively greater proportion of deaths from all causes. Moreover, alcohol consumption has been shown to account for 83% of the variance in such deaths over the period in question (Seeley, 1960).

It is estimated that in 1968 about 617,000 people, or 5.45% of the drinking population were consuming sufficiently large amounts of alcohol to increase the risk of hepatic disease; that of these drinkers 300,000, (2.6% of the adult population aged 20 years and older) were suffering from alcoholism; and approximately 1,000 alcoholics died from cirrhosis (A.R.F. 1957-71).

If the increase in per capita consumption of alcohol continues unchecked in Canada, as well as those other countries where drinking is culturally accepted, it seems likely that their levels of cirrhosis mortality will ultimately reach that in France of approximately 45 per 100,000 adults (de Lint and Schmidt, 1971).

RELATIONSHIP BETWEEN PER CAPITA CONSUMPTION AND HAZARDOUS DRINKING

An understanding of the relationships that exist between per capita consumption, the distribution of alcohol consumption amongst drinkers and the proportion of drinkers who drink hazardous amounts, aids our understanding of some of the epidemiological aspects of alcoholic liver disease.

First, it is necessary to define hazardous drinking and alcoholism. Hazardous drinking has been defined quantitatively as a daily intake of 100 ml. (80 gm.) or more of absolute alcohol. One hundred ml. of absolute alcohol is contained in approximately 9 oz. of spirits, 21 oz. of wine (15% alcohol by volume) or 72 oz. of beer. Consumption at or above this level has been described as "hazardous" because it is the point at which there is a significant increase in the risk of drinkers developing cirrhosis (Péquignot, 1958). Within a population of hazardous drinkers there are those who are defined as alcoholic because their drinking is associated with a disturbance of their physical, mental and/or social health.

With these operative definitions in mind, it is important to understand that in a drinking population, the distribution of daily alcohol consumption is not bimodal, as might be suggested by concepts of alcoholism that view the alcoholic population as entirely distinct and separate from the population of "normal" or "social" drinkers. Rather it is a continuous, unimodal, skewed distribution of logarithmic normal type, and can be determined for any drinking population from the per capita consumption by utilizing Ledermann's equation (Ledermann, 1956)

$$t_s = aZ_s + \theta$$

Ledermann derived his equation from empirical observations of the distribution of alcohol consumption in populations with quite different per capita values — France, Finland, Sweden and the U.S.A. — and its application to Canadian data has since been validated independently (de Lint and Schmidt, 1968).

The logarithmic normal distributions of daily alcohol consumption for three populations of drinkers is illustrated in Figure 1. One population has a low level of consumption, 7.5 litres per year (equivalent to 10 ml. absolute alcohol per day); one has an intermediate level, 17.5 litres per year (equivalent to 48 ml. per day); and one has a high level, 30 litres per year (equivalent to 82 ml. per day). The following points should be noted from Figure 1.

1. Although "normal" and "hazardous" drinking have been defined empirically as different on the basis of clinical observations, they are part of the same continuous, unimodal distribution of any drinking population.

2. The shape of the distribution curve changes when the mean consumption alters so that as the per capita consumption increases there is an increasing proportion of drinkers consuming at or above 100 ml. of alcohol per day.

It is important to consider this second point further. Using the Ledermann equation it is possible to calculate for populations with different per capita consumption levels the percentages of those populations consuming at or above 100 ml. of alcohol per day. The relationship of these variables is curvilinear, indicating that the percentage of hazardous drinkers rises at a faster rate than the per capita consumption so that over most of the range, a given increase in per capita consumption is accompanied by an even greater increase in the prevalence of hazardous drinkers (Figure 2).

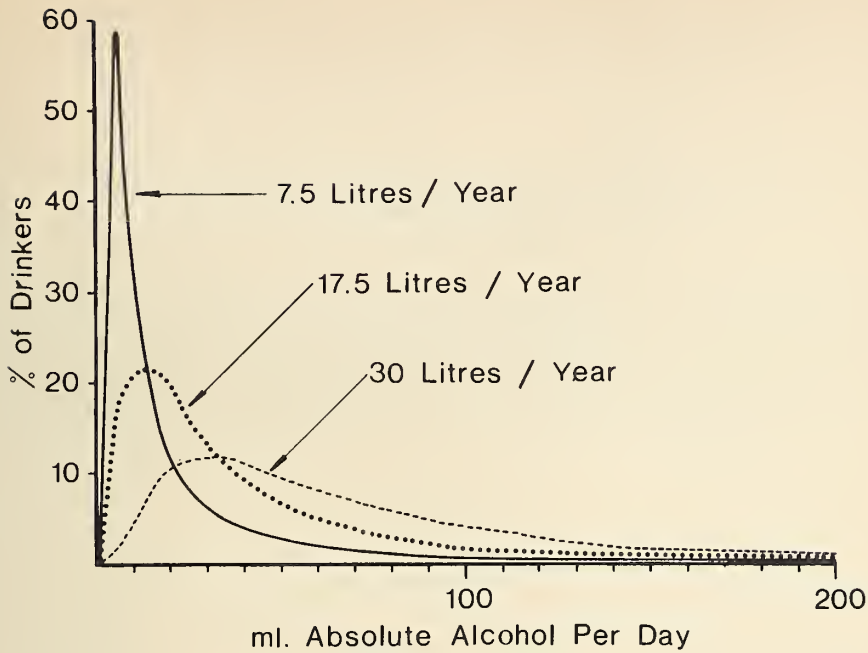


Figure 1. *Distribution curves of daily alcohol intake for populations with levels of annual per capita consumption of 7.5, 17.5 and 30 litres of absolute alcohol per year.*

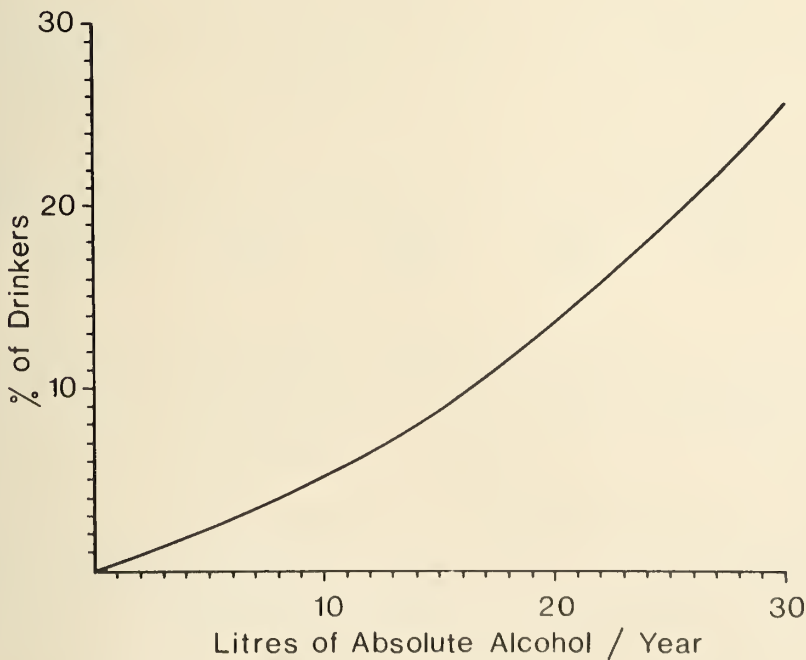


Figure 2. *Relationship between various levels of annual per capita consumption of alcohol and the percentage of drinkers whose consumption is equal to or in excess of 100 ml of alcohol per day.*

For example a 50% increase in per capita consumption from 20 to 30 litres is accompanied by slightly more than a 100% increase in the prevalence of hazardous drinkers.

RELATIONSHIP BETWEEN PER CAPITA CONSUMPTION AND DEATHS FROM CIRRHOSIS IN ONTARIO

Ontario statistics exemplify the relationship that exists between per capita consumption of alcohol and deaths from cirrhosis. The changes in per capita consumption and deaths from cirrhosis of the liver from 1928 to 1967 are illustrated in Figure 3. The per capita consumption rose from 1.77 litres in 1933 to 8.91 litres in 1967. The cirrhosis death rate rose from 3.8 per 100,000 in 1930 to 13.2 in 1967 (Popham, Schmidt and de Lint, in press).

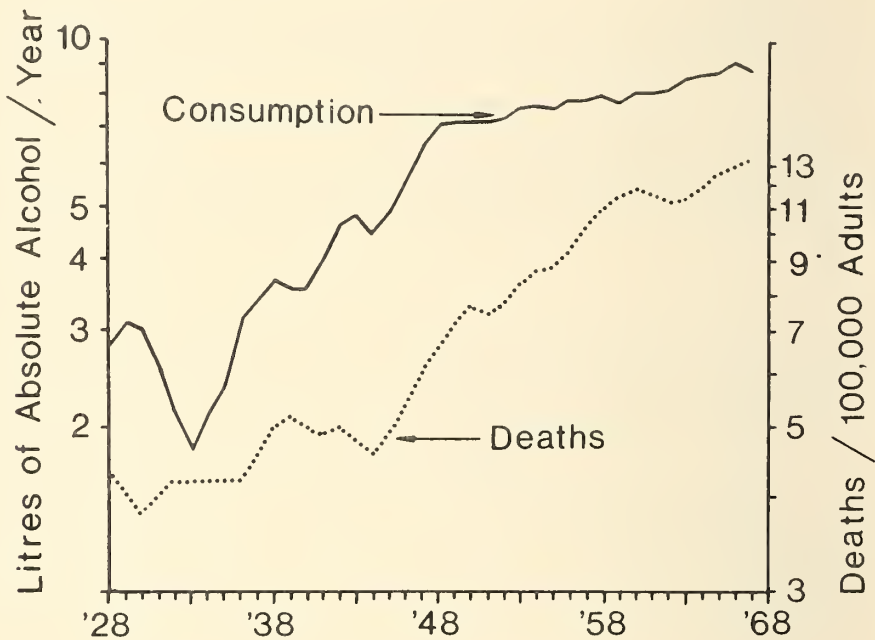


Figure 3. Variations in per capita alcohol consumption and adult death rate from cirrhosis in Ontario between 1928 and 1967.

In Figure 4 the same information for cirrhosis death rate and per capita consumption has been plotted against one another. There is a high, positive and curvilinear correlation. Changes in alcohol consumption account for about 83 per cent of the variation in cirrhosis death rate. The information suggests that the death rate from non-alcoholic cirrhosis is approximately 3 per 100,000, i.e. the point on the vertical axis where the curvilinear line intersects and the alcohol consumption is zero. If it is assumed that the adult death rate from non-alcoholic cirrhosis has remained relatively stable during the period 1930 to 1967 then in 1967 alcoholic cirrhosis accounted for 65% of the deaths from cirrhosis in Ontario. All these calculations have been based on age-sex standardized

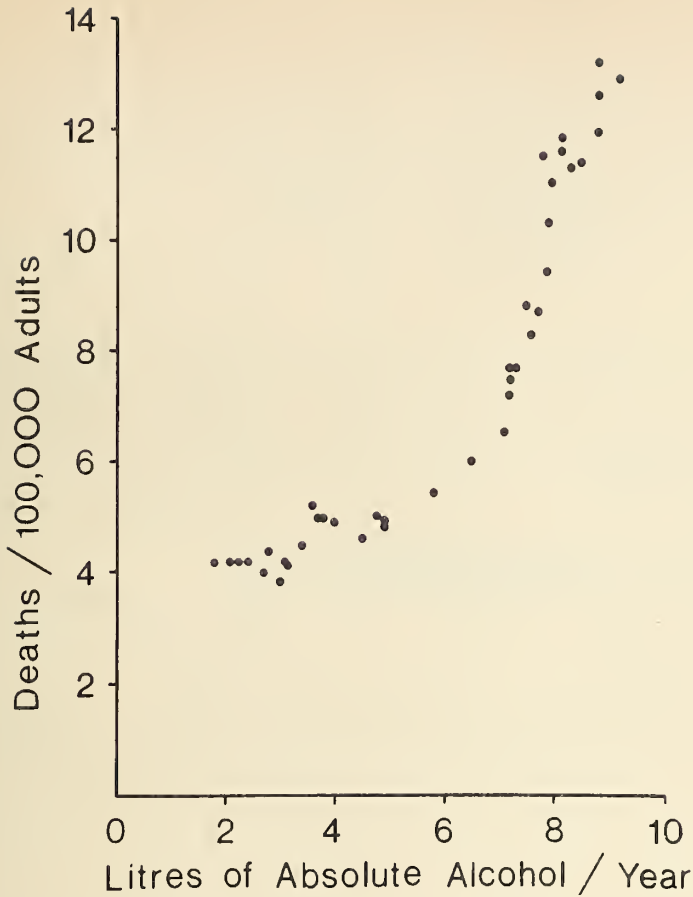


Figure 4. Relationship between per capita alcohol consumption and adult death rate from cirrhosis in Ontario.

rates of death from cirrhosis of the liver. The curvilinear relationship between death rate and per capita consumption may be explained in part by the curvilinear relationship that exists between per capita consumption and the percentage of hazardous drinkers.

AETIOLOGY OF ALCOHOLIC CIRRHOSIS

Epidemiological studies have provided some of the pieces in the incomplete puzzle of the aetiology of alcoholic cirrhosis and indicate possible areas for speculation and research.

The following summarizes present knowledge of some of these potential aetiological factors.

Quantity of Alcohol Consumed Daily

The work of Péquignot (1958) revealed that the risk of developing alcoholic cirrhosis increases measurably when the consumption exceeds 100 ml. of absolute alcohol per day and becomes much greater when the consumption exceeds 200 ml. per day. However, it has also been noted that alcoholic women drink less per day than alcoholic men and develop cirrhosis at lower levels than men (Wilkinson, Santamaria and Rankin, 1969).

Duration of Drinking

It has been found that alcoholics with cirrhosis have drunk hazardously and at higher levels for a longer period than those without, (Lelbach 1967a) and that alcoholic women develop cirrhosis after a shorter period of hazardous drinking than alcoholic men (Wilkinson, Santamaria and Rankin, 1969).

Pattern of Drinking

It has been observed that cirrhosis occurs more frequently in inveterate drinkers than in bender drinkers who alternate bouts of excessive drinking with periods of moderate drinking or complete abstinence (Wilkinson, Santamaria and Rankin, 1969). This difference can be explained on the basis of a much shorter period of actual hazardous drinking in bender than habitual drinkers so that the former group drinks less over a lifetime than the latter.

Type of Alcoholic Beverage

There is no epidemiological evidence that one type of beverage is more likely to produce cirrhosis than another (Lelbach 1967b; de Lint, Schmidt and Popham 1970).

Malnutrition

Although malnutrition can contribute to the production of acute hepatic disease in the alcoholic, no differences in nutritional history have been demonstrated between alcoholics with or without cirrhosis (Péquignot 1958) except for the longer period of hazardous drinking and therefore presumed longer period of consequent malnutrition in those with cirrhosis compared to those without.

Ethnicity

Although there are some variations in cirrhosis death rates between countries with similar levels of per capita consumption of alcohol, no other evidence has been found for racial determinants of alcoholic cirrhosis.

Sex

Women appear to be more susceptible to alcoholic cirrhosis than men (Wilkinson, Santamaria and Rankin, 1969), as they are to some other diseases of the liver such as cryptogenic cirrhosis, chronic active hepatitis, primary biliary cirrhosis and fulminant cirrhosis. Attention has already been drawn to the fact that alcoholic women develop cirrhosis at a lower level of consumption and after a shorter period of excessive drinking than alcoholic men. In addition, cirrhosis has been found to occur approximately twice as commonly in alcoholic women than in alcoholic men. In a study by Wilkinson and her

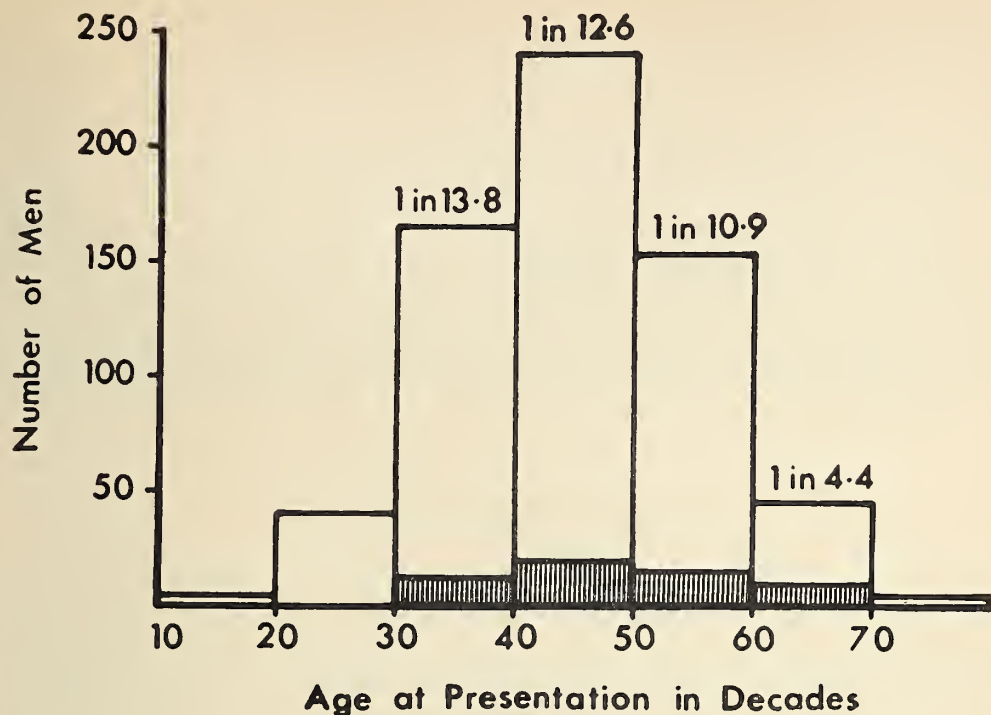


Figure 5. Prevalence of cirrhosis in alcoholic men (cirrhotic patients shown hatched).

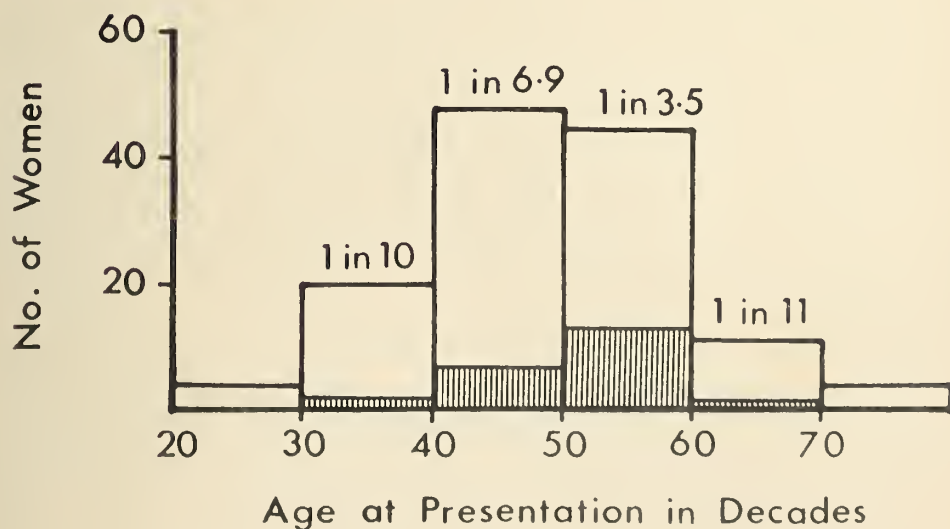


Figure 6. Prevalence of cirrhosis in alcoholic women (cirrhotic patients shown hatched).

co-workers it was found that the prevalence of cirrhosis amongst alcoholic men increased slightly with increasing age. (See Figure 5.) By contrast, amongst the women in this study, cirrhosis showed a marked increase in prevalence in the fifth and sixth decades when it is possible that hormonal changes may have in some way constitutionally predisposed them to an increased risk of developing the disease. (See Figure 6.)

These observations by no means explain the causation of cirrhosis in the alcoholic, for although acute liver disease is an almost invariable complication of alcoholism, cirrhosis still occurs sporadically, suggesting the sporadic occurrence of some other factor or factors, perhaps some underlying genetic or acquired predisposition. Results of studies of cardiovascular and cerebral diseases in alcoholism also suggest the possible importance of genetic or constitutional factors in the aetiology of some chronic physical complications (Wilkinson, Kornaczewski, Rankin and Santamaria 1971; Horvath, in press.)

INTERACTION OF HEPATIC DISEASE PROCESSES IN THE ALCOHOLIC

At this stage it seems both useful and necessary to speculate further on the potential importance of the interaction of alcohol excess and malnutrition with other factors in the aetiology of alcoholic cirrhosis. Usually when the aetiology of alcoholic cirrhosis is under consideration, the focus is rather unrealistically on the response of the normal liver to a hostile environment of alcohol excess and associated malnutrition. In reality, the alcoholic is exposed to at least the normal risks of developing inherited or acquired forms of non-alcoholic hepatic disease, and these disease processes, in turn, might either modify or be modified by hepatic disease attributable to alcohol excess with or without associated malnutrition. From this standpoint, four categories of hepatic disease processes can be identified in the alcoholic:

1. *Alcoholic hepatic diseases apparently due to alcohol excess with or without malnutrition.* This group includes biochemical abnormalities detectable only by electron-microscopy, acute alcoholic hepatitis and cirrhosis.

2. *Inherited hepatic disease in which alcohol excess and possibly associated malnutrition can apparently affect the degree of genetic expression.* The known diseases in this category are haemachromatosis (Williams, Scheuer and Sherlock, 1962) and cutaneous porphyria (Holmes and Barnes, 1965). Although both can occur in people who are either abstinent or moderate drinkers, heavy drinking is a common finding. If the potential mechanisms of hepatic damage in these two diseases are either triggered or facilitated by alcohol excess, is there any reason why there cannot exist a genetically determined cirrhotic mechanism which is also activated by alcohol excess? In Australia and Austria an association has been found between blood group A and portal cirrhosis (Billington, 1956; Wewalka, 1960).

3. *Hepatic diseases due to the misuse of psychoactive drugs other than alcohol.* Excessive drinking and the misuse of psychoactive chemicals other than alcohol are frequently associated in the same individual. Hepatic disease could be caused by the use of a hepatotoxic drug or the hepatotoxicity of substances injected in association with a drug, e.g. other compounds, and bacteria and viruses, particularly those of Australia antigen positive and negative hepatitis. What part such hepatic diseases might potentially play in the aetiology of cirrhosis in the alcoholic is difficult to say. However, this association of alcoholism with other forms of drug use already appears to be influencing the pattern and frequency of hepatic disease in hazardous drinkers.

4. *Hepatic disease processes not caused by the misuse of alcohol or other psycho-active chemicals.* Finally, there are those disease processes which may lead to the development of cirrhosis in the non-alcoholic, e.g. viral hepatitis and autoimmunity. The question arises as to what extent these processes when they occur in an alcoholic may modify or be modified by alcohol excess and result in an increased prevalence of "alcoholic" cirrhosis?

Although hepatitis-associated antigen is rarely found in patients with alcoholic cirrhosis (Wright, McCallum and Klatskin, 1969), Pettigrew and his co-workers found evidence of past infection with hepatitis B virus in all of 11 patients with chronic alcoholic liver disease. They considered their findings indicated that the virus was an aetiological factor in alcoholic cirrhosis (Pettigrew, Russell, Goudie and Chaudhau, 1972).

HOW CAN THE MORBIDITY AND MORTALITY FROM ALCOHOLIC CIRRHOSIS BE REDUCED?

Hopes for the reduction in morbidity and mortality from alcoholic cirrhosis lie in three general directions. The first is theoretical and would evolve out of learning how to prevent the cirrhotic process. The other two are both practical and achievable. First, a reduction of morbidity and mortality by successfully rehabilitating alcoholics either with or without cirrhosis, and secondly, lowering the prevalence of alcoholic cirrhosis by lowering the per capita consumption of alcohol.

In Canada, where approximately 80 per cent of the population drink, relative cost of alcohol appears to be the dominant factor affecting per capita consumption. This is illustrated in Figure 7, which shows the relationship between per capita consumption and

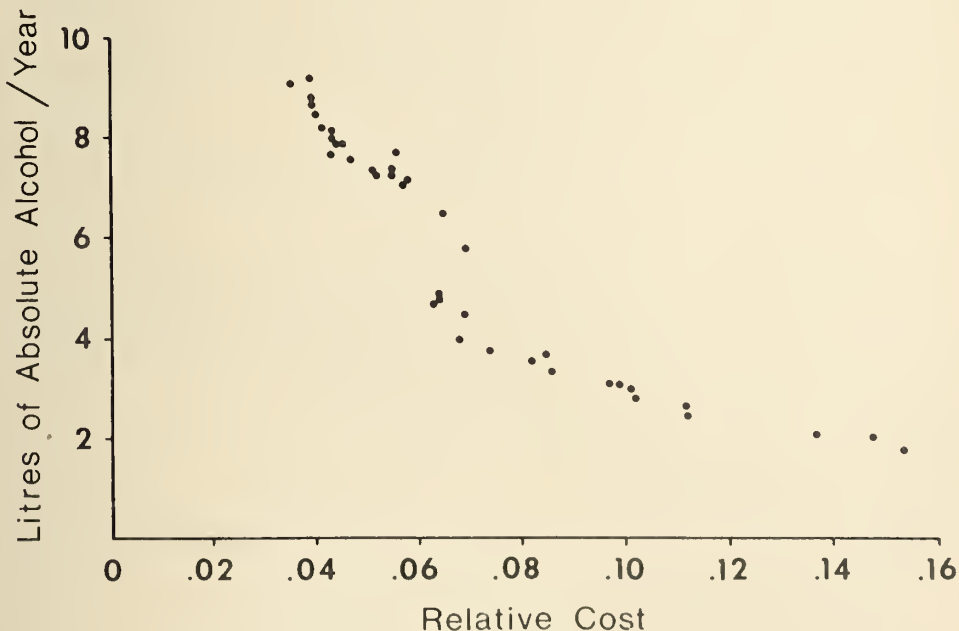


Figure 7. Relationship between per capita alcohol consumption and relative cost of alcoholic beverages in Ontario.

relative price of beverage alcohol during the period 1928-1967 (Popham, Schmidt and de Lint, in press). It can be seen that consumption rises in a somewhat curvilinear fashion as the relative price falls. Relative price has fallen more or less progressively since 1933. This knowledge provides a basis for effectively countering what is currently a mounting problem of alcoholic liver disease. Further increases in the prevalence of alcoholic hepatic disease could be prevented by stabilizing the present relative cost of alcohol at its present level and thereby stabilizing per capita consumption. Alternatively, attempts could be made to decrease the prevalence of alcoholic hepatic disease by increasing the relative cost and thereby lowering the per capita consumption. The potential difficulties of reducing the per capita consumption by this means are formidable. However, attempts to stabilize at the present level of per capita consumption would seem both a feasible and worthwhile first step.

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Liver Architecture and Microcirculation

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INTRODUCTION

The subject to be presented is indeed an old one and a historical note will demonstrate that our great pioneers in anatomy and physiology, centuries back, have described the hepatic tissue correctly, that is, the parenchyma as organized around the supplying, the nutrient, vessels.

The circulation of the liver under the microscope had been noticed first by Malpighi in 1666; he described the hepatic lobules (acini) as "appended to the extremities of vessels." Malpighi also quoted Harvey's account of the developing liver as supporting his own view of the adult one. In 1651 in his book *On the Generation of Animals*, Harvey wrote "that on the seventh and tenth days of fetus development the parenchyma of the liver grows upon the branches of the umbilical vessels just as grapes upon the bunch, buds upon twigs and the beginning spike upon the grain stalk". Harvey's and Malpighi's concept of hepatic structure and microcirculation was generally accepted for almost two centuries because we find it still in Johannes Müller's *Textbook of Physiology* in 1844. Müller, the father of modern physiology, in his study of the structure and function of glands, saw in the acini of Malpighi "an agglomeration of primitive cells that secrete the bile into the ductules; the cells fill the spaces between the vascular loops of the lobule."

The *in vivo* study of the liver aimed particularly at its microcirculation has been initiated in the United States by Knisely (1936, 1939), Bloch (1940, 1955), Wakim and Mann (1942), in Germany by Löffler (1925), and Ellinger and Hirt (1929), in Britain by Macgrath, Andrews and Wenyon (1949) and by Seneviratne (1949), in Canada by Rappaport and associates (1958, 1966), in France by Chenderovici and Caroli (1962), in Sweden by Hanzon (1952), and in Japan by Nakata (1955, 1967).

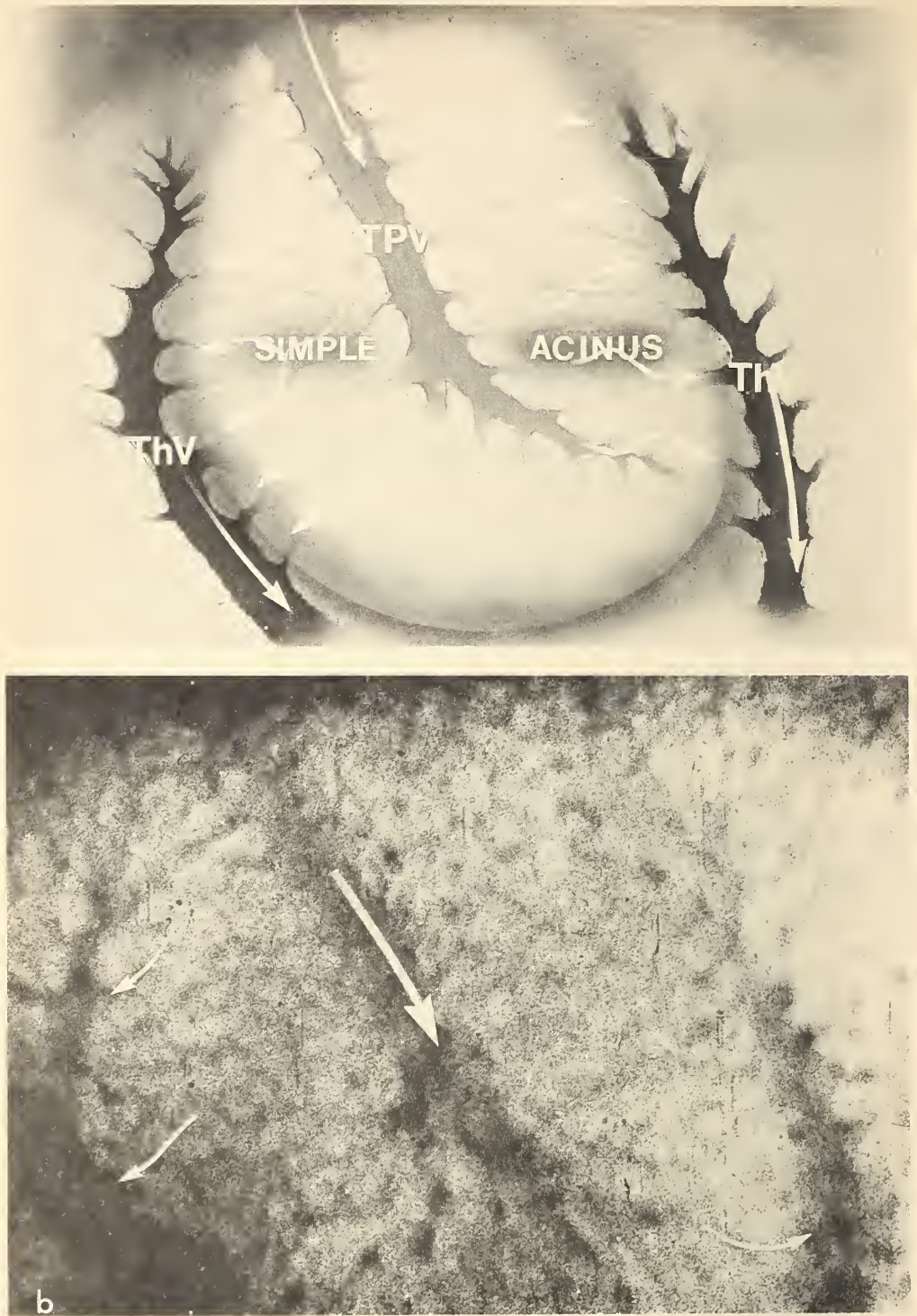


Figure 1 a) *Diagram of structural hepatic unit. A simple liver acinus is shown with its terminal portal venule and two draining hepatic end-venules. TPV = Terminal portal venule; ThV = Terminal hepatic venule. b) A simple acinus. A microscopic mass of parenchyma is photographed in vivo in the transilluminated liver of a rat. The orientation of the venous vessels supplying and draining the acinus is evident from the above diagram. (From Rappaport, 1973, courtesy Academic Press, N.Y.)*

However, almost all authors of our century found themselves constrained to report their findings using the hexagonal lobule of Kiernan (1833) as a hepatic unit. This led to a distorted description of the microcirculatory phenomena observed *in vivo*, in flagrant contradiction to the basic law of circulatory physiology. This law indicates that due to a pressure gradient blood flows from the afferent end vessels through the capillaries toward the peripheral and efferent end venules. However, in the description of the hexagonal lobule one finds that:

1. The lobule is supplied from a number of portal and arterial vessels and thus has no microcirculatory unity.
2. The supplying vessels are considered to be at the periphery of the lobule whereas from a circulatory viewpoint they are the center of the lobular microcirculation.
3. Each supplying vessel delivers its blood into two hexagonal fields, hence the prerequisite of unity is undone six times. Such a vessel viewed from the center point of a hexagon appears as a miniature rivulet with one shore only.

To avoid this unrealistic viewpoint we will describe the hepatic structural units in accordance with the direction of flow in the microvasculature that sustains them.

THE *IN VIVO* STRUCTURAL UNITS OF THE LIVER AND THEIR MICROVASCULATURE

Structural Units

The *simple liver acinus* (Fig. 1a, b) represents a small parenchymal mass *irregular* in size and shape arranged around an axis consisting of a terminal hepatic arteriole (THA), terminal portal venule (TPV), bile ductule(s) (BD), lymph vessels, and nerves which grow



Figure 2 Large and small hexagons in the vinylite cast of the portal vessels of a human liver. Polygons of various size and shape are formed by the portal vessels coursing around central hepatic venous branches of corresponding size (not shown in this specimen). Polygon 1 corresponds to a microscopic hexagonal field $\times 14$. (From Rappaport, 1963, courtesy Academic Press, N.Y.)

out together from similar preterminal structures in a small *triangular* portal field (Rappaport, Borowy, Loughheed and Lotto, 1954).

An acinus lies between two or more terminal hepatic venules (ThV), the so-called central veins, with which its vascular and biliary axial channels interdigitate. The interdigitation of terminal branches originating from three or more triangular portal spaces around one ThV may create a vascular pattern simulating a hexagon. The interdigitation of afferent vessels with the efferent ones is the result of developmental ingrowth of the hepatic parenchyma with its supplying vessels and bile ductules into the omphalo-mesenteric venous plexus, the remnant of which are the hepatic veins. Interdigitation is therefore present at macroscopic level too (Fig. 2), and the polygonal areas delimited by the afferent vessels are devoid of structural microcirculatory and secretory unity. Shortly, the "*hexagonal lobule*" is not a unit.

The fascination with the hexagonal lobule persisted for one and a half centuries, and the question of how and where the liver acini fit into the hepatic hexagons must be answered. The simultaneous injection of two differently colored gelatin masses into the first lobar branches of the portal vein under 200 mm H₂O pressure demonstrates that an acinus occupies sectors only of two adjacent hexagonal fields (Fig. 3). Thus the area of one hexagon contains parts of several acini organized around THA, TPV and BD that interdigitate with the ThV ("central vein").

Using similar technic and differently colored vinylite masses for the injection of all afferent and efferent vessels, one can obtain a vascular cast of the entire vasculature of the liver. The cast demonstrates the interdigitation of the supplying with the draining hepatic vessels and a polygonal orientation of the former around the latter (Fig. 4a, b).

A simple liver acinus is subdivided into three circulatory zones (Fig. 10) which surround the axial structures like layers of a bulb. The cells in zone 1 are situated close to the supplying vessels; they are bathed by blood of a composition similar to that in the afferent vessels. The cells in zone 3 are the most distant from their own supplying vessels as well as from those feeding the neighbouring acini. Hence, zone 3 is situated at the microcirculatory periphery of the acinar unit and receives blood that has already exchanged gases and metabolites with cells in zones 1 and 2. Zones 3 of several adjacent acini lie close to a ThV, their common drainage center, *i.e.*, at the most peripheral parts, *the acra* of the hepatic microcirculation. It is evident that cells of zone 3 are most sensitive to damage through ischemia, anoxia, congestion, and nutritional deficiency (Rappaport, 1963, 1969).

The simple liver acinus is also the *secretory* unit of the liver; the bile produced by it is delivered into the terminal bile ductule, part of the same portal triad. The watershed of bile flow is the dividing line of two adjacent acini.

The *complex acinus* (Fig. 5a) is a microscopic clump of tissue composed of at least three simple acini and a sleeve of parenchyma around the preterminal portal, arterial, and biliary branches, lymph vessels, and nerves giving origin to the terminal axial channels of the simple acini that constitute this larger unit. The preterminal portal branch ramifies in three directions. Each of its terminal branches forms the axis of a simple acinus, whose cells occupy the intersinusoidal spaces in the various microcirculatory zones. The sinusoids drain into at least two terminal hepatic venules situated between zones 3 of adjacent acini. A similar distribution of vessels is seen *in vivo* in the transilluminated liver of rodents (Fig. 5b). There is also a distinct clump of parenchyma surrounding the preterminal vascular and biliary structures of the complex acinus. This sleeve of parenchyma consists of tiny acini (acinuli) that are supplied by small portal venules and by arterioles branching off the preterminal vessels in the triangular portal spaces. Such small



Figure 3 Subdivision of the hexagonal lobules into hepatic structural and functional units (liver acini). The structural units (LA) are small berry-like parenchymal masses around the trio of terminal branches of portal vein, hepatic artery and bile duct branching out from a small triangular portal space. L^1A^1 = horizontal section through a red colored hepatic unit, extending from one terminal hepatic venule ("central vein") to another. (Rappaport, Borowy, Lotto and Loughheed, 1954.)



Figure 4 a) Vinylite cast of human liver. The right lobar branch of the portal vein has been injected with green colored vinyl acetate, the left one with red, and the hepatic vein with yellow. Note that in the left half of the figure the area around the yellow hepatic venule ("central vein") is delimited by red and green portal branches.



Figure 4 b) Three colored vinylite cast of portal and hepatic veins in a rat. Terminal vessels of the left and right lobar portal branches (red and greenish) are seen under the bio-objective microscope in the right half of the field, on each side of the terminal hepatic venous branches springing in vertical direction from a preterminal parent branch (light blue). Some of the terminal branches appear in the picture as bluish dots only, $\times 20$. (From Rappaport, 1963, courtesy Academic Press, N. Y.)

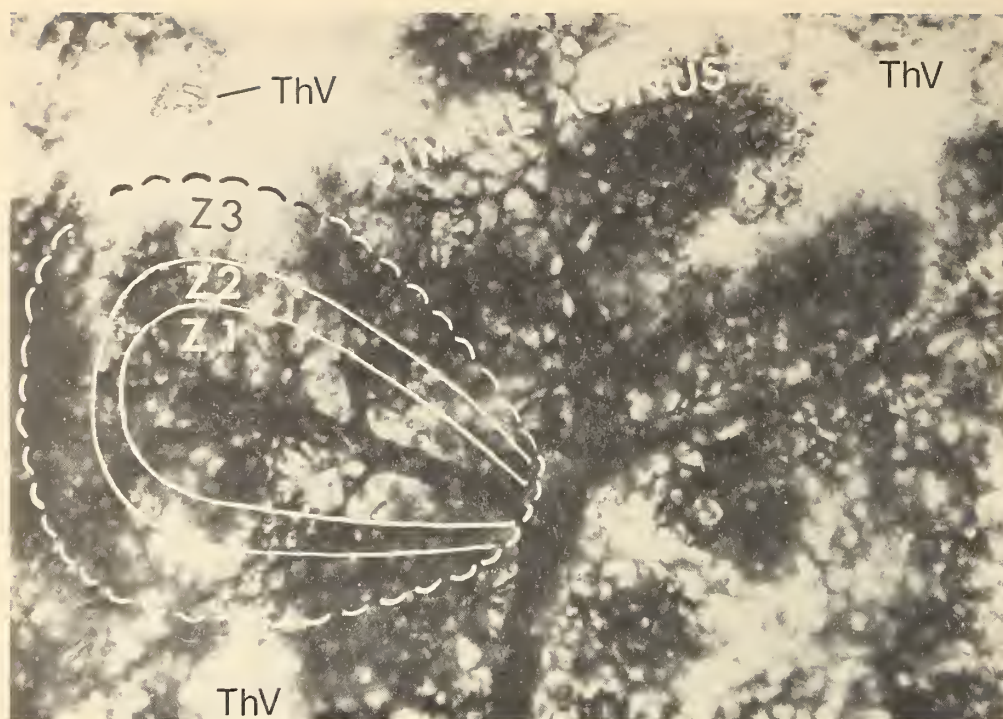


Figure 5 a) *Human complex acinus composed of simple acini. Three terminal portal venules branch out from a preterminal parent stem; each of them together with its sinusoids irrigates a simple acinus. In the left acinus the zonal arrangement of the hepatocytes situated in the intersinusoidal spaces is indicated. Zone 1 (Z_1) harbors the tissue close to the terminal afferent vessels. Cells at the microcirculatory periphery (Z_3) are more vulnerable to ischemia and dietary deficiency. Human liver injected with india ink; thick cleared section $\times 60$. (From Rappaport, 1973, courtesy Academic Press, N.Y.)*



b) *Complex acinus in rat's liver. Three terminal portal branches originating from a preterminal vessel are seen in this enlarged frame of a movie taken of the in vivo transilluminated liver $\times 80$. (From Rappaport, 1972.)*

vessels have been demonstrated radiologically by Daniel and Prichard (1951). A cut through the complex acinus shown in Figure 5a at the level of division of the preterminal vessel into its terminal branches would have to be inclined toward the left in order to lay bare in one cross section all three terminal branches cropping out from the vessels in the triangular portal space as shown in Figure 10. The latter are the nutrient vessels of the entire complex acinus. The subdivision of the complex acinus into microcirculatory zones can be obtained by extending the zonal subdivision from the left simple acinus to the other acini. Zone 1 would appear then as a trident while zone 3 would best be simulated by three adjacent and opened umbrellas whose central sticks form the trident representing zone 1. Lesions occupying such three vaulted areas can be seen in the liver damaged by a fair degree of ischemia or nutritional deficiency.



Figure 6 *Human acinar agglomerate.* An acinar agglomerate is formed of complex acini. The entire clump of tissue is comparable to a crown of a tree, because its portal vessels were injected with india ink. The axes of the complex acini are formed by preterminal afferent vessels (1). Each preterminal vessel divides into 3 terminal branches (2) which arch out tridimensionally and form the axes of simple acini. Human liver injected with india ink, 100 μ thick cleared section, $\times 30$. (From Rappaport and Hiraki, 1958.)

The complex acini are parts of the largest microscopic acinar unit, the acinar agglomerate, consisting of at least three complex acini (Fig. 6).

Microvasculature

Each liver acinus is supported by a microvascular framework consisting of hepatic arteriole(s), terminal portal venule, a glomus of sinusoids, terminal hepatic venules and their nerves forming together with lymph vessels (Rappaport 1973):

THE MICROCIRCULATORY HEPATIC UNIT

(a) *The hepatic arterioles* range from $100\ \mu$ to $50\ \mu$ in diameter. The terminal arterioles vary in diameter between $50\ \mu$ and $15\ \mu$ and they still have an elastica interna and a single layer of smooth muscle cells (Burkel, 1970). These arterioles and their capillaries ($10\text{--}8\ \mu$ wide) lace a dense plexus around the bile ductules (Fig. 7); some of the capillaries have a 'precapillary sphincter' (Fig. 8b) (Rhodin, 1967) and their *non fenestrated* endothelium has a basement membrane (Fig. 8a). Efferent arterioles and venules originate from the periductular plexus where the blood pressure has been substantially lowered; they join the sinusoids or the TPV. Some arterioles bypass the periductular plexus and empty directly with strongly pulsating jets into the TPV (Fig. 7) as can be noted in the film "Normal Microcirculation of the Mammalian Liver" (Rappaport, 1972). All arteriolar openings are found *in zone 1 only*.



Figure 7 *Microcirculatory hepatic unit. The unit consists of: (a) the terminal portal venule (TPV) with the sinusoids branching off it and forming a glomus; (b) the hepatic arteriole (THA) lacing with its branches a plexus around the terminal bile ductule (BD). The arterioles empty either directly (1) or via the peribiliary plexus (2) into the TPV and sinusoids. The sinusoids run along the outside of cell plates and cords inside which are the capillaries of the hepatic secretory and excretory system. The glomus of sinusoids is drained by at least two terminal hepatic venules (ThV); Ly = lymphatics. (From Rappaport, 1973, courtesy Academic Press, N.Y.).*



Figure 8 a) *Terminal hepatic arteriole.* The arteriole (ha) is of simple architecture; it consists of endothelium (E) and of tunica media (TM) formed by one layer of smooth muscle cells; there is a common basement membrane (BM) between the two. Projections from the endothelium form myoendothelial contacts (see arrow in inset). The surrounding cells of the portal space are tightly packed against the media (TM). N = unmyelinated nerve fibre ($\times 3600$, inset $\times 7100$).

Figure 8 b) *Precapillary sphincter.* A capillary (C) branches off from an arteriole (HA) about $60\ \mu$ in diameter. Smooth muscle cells of the arteriolar media form a cuff surrounding the endothelium of the capillary. The muscular cuff represents a precapillary sphincter (PCS); sphincter and endothelium together constitute a precapillary. The smooth muscle cells of the sphincter are in membranous contact with each other, with the endothelium (arrows), and with unmyelinated nerve fibres (N). ($\times 4300$, courtesy of Burkel, 1970).

(b) *The terminal portal venules* are about $20\ \mu$ wide; their wall does not have smooth muscle fibres, only endothelium, basement membrane and scant connective tissue. Consequently at the site of the origin of the sinusoids there are no muscular inlet sphincters, only large endothelial cells which by swelling or shrinking can regulate inflow of blood into the sinusoids (McCuskey, 1966).

(c) *The sinusoids*, the venous capillaries of the liver, have an average width of $14\ \mu$, but they can widen and permit the simultaneous passage of 4 erythrocytes. The length of the sinusoid varies with the species and is $250\ \mu$ in the rat. The endothelial lining of the sinusoids is thin and fenestrated for an easy passage of the plasma fluid into and from the Disse space surrounding the sinusoids and continuing into similar space around the TPV and ThV.

(d) *The terminal hepatic venule* is at the *terminal* of the hepatic microcirculation and it is misleading to call this peripheral venule "central vein". The wall structure of the ThV is similar to that of the TPV but has fewer vascular pericytes and reticular fibres. Its endothelial lining has a basement membrane.

Regulation of the Hepatic Microcirculation

The arterioles with their muscular wall containing unmyelinated nerve fibres are the chief regulators of the intrahepatic blood flow. The pressure in the arterioles has not been measured yet but must be assumed to be similar to that in any other arteriole (30-35 mm Hg). The pressure in the TPV is low (3 mm Hg). When the arterioles in a microscopic area are constricted, portal blood can still flow due to the pressure gradient between TPV (50 mm H_2O) and ThV (5 mm H_2O). Upon opening of their sphincters, the arterioles jet their blood into the sinusoids and increase the *vis a tergo* of the venous flow therein and the mixed arterial-portal blood is swept ahead through the sinusoids into the ThV. The activity of the arterioles and of the endothelial inlet and outlet gates of the sinusoids is intermittent, thus altering the movement of the plasma from and into the sinusoids. *Outward motion* of plasma occurs upon opening of the arterioles, narrowing of the sinusoidal outlet, and rise in hepatic venous pressure. *The inward movement* of plasma into the sinusoidal lumen takes place upon narrowing of the sinusoidal inlet gates, constriction of the arterioles, and widening of the sinusoidal outlets. Glycogenolytic hormones, K, and ATP (McCuskey, 1966) relax the arteriolar sphincters. The swift current of arterial blood moves glucose out of the liver quickly and at the same time provides an arterial pO_2 necessary for increased metabolic activity, *i.e.* gluconeogenesis (also from K-lactate) and ATP formation. Stimulation of the sympathetic hepatic nerve plexus constricts the arterioles. It is also to be expected that other intermediary metabolites and electrolytes have their local regulatory effect on the microcirculation. Adjustment of the microcirculation to hepatic metabolism occurs further through bile secretion. The injection of Na dehydrocholate (Decholine) causes choleresis together with increased hepatic arterial flow (Schwiegk, 1932; Duffin, Llewellyn Thomas and Rappaport, 1965). As blood flow through the periductular arterial plexus is increased, the efferent arterioles of this plexus augment arterial inflow into the TPV and sinusoids. The conjugated bile salts (Na taurocholate and Na glycocholate) reabsorbed during digestion may have similar effects although to a lesser degree. Still, regulation of the hepatic microcirculation through bile salts may provide adjustment of blood flow to the liver during increased metabolic activity in the postabsorptive phase of digestion.

All these data indicate that the merging of the arterial and portal stream in the sinusoidal delta is well regulated, that the arterial blood under high pressure does not drop like one large waterfall into the low pressure level of the sinusoidal bed. The arterial stream is distributed through millions of arteriolar rivulets into the microcirculatory hepatic units of the acini, increasing or decreasing their arterial supply and adjusting it to their individual metabolic activity. The fully oxygenated blood of the arterioles raises the O_2 tension in the periportal area. In the parenchyma of the acinus is created a pO_2 gradient that decreases towards the ThV. The periportal microenvironment is therefore different from the perivenular one (Miller and Kessler, 1973). In establishing different living conditions for cells in two neighbouring areas *the arterioles assume the organizing role within the liver acinus*. Lack of good arterial supply is now shown experimentally to be the cause of persistent fetal structure and of poorly organized hepatic drainage, *i.e.*, of congenital biliary atresia. It has been demonstrated by Morgan, Rosenkrantz and Hill (1966) who ligated the H.A. in the fetus of the rabbit and produced biliary atresia experimentally.

Metabolic Zones

Different arterial irrigation and pO_2 in zones 1 and 3 create microenvironments suitable for specific enzymic activities. From the data collected in the literature of histochemistry and enzymology and tabulated in Figure 9 we conclude that in the liver acinus there is metabolic organization in close connection with the direction of blood flow in the unit. As indicated by the high level of UDP-glucose: α -4 glucosyltransferase (UDPGGT), phosphorylase and glucose 6-phosphatase activities, the cells in zone 1 are geared to glycogen synthesis (Sasse, 1969, Sasse and Köhler, 1969) and glycogenolysis. In these cells containing numerous long mitochondria, oxidative processes operate at a high level via the Krebs cycle. There too, is increased activity of respiratory enzymes such as succinic dehydrogenase and cytochrome oxidase. The abundance of lysosomes rich in acid phosphatase facilitates a higher rate of pinocytosis and uptake of materials from the nutrient-laden portal blood. Le Bouton (1968) has demonstrated that zone 1 is the prime area of protein metabolism and formation of plasma proteins. He established that length and width of the active area coincides with the extent of zone 1.

Zone 3 is the site of NAD and NADH tetrazolium reductase activity, of glycogen storage, of fat and pigment formation. With increased fat formation the lipid laden cells are seen also at the perivenular site where zones 3 of several adjacent acini adjoin. Enzymatic specificity and metabolic heterogeneity of cells in different circulatory zones must be implicated in the selective toxic injury of cells in different parts of the liver acinus (Stoner, 1956; Wilson, 1958). This differing degree of susceptibility to damage by anoxia or malnutrition in different zones enabled us to delimit these zones before their enzymic pattern was established. However, the outlined enzymic activity should not be regarded as a fixed map. The hepatic cells are capable of multiple metabolic functions; pathologic changes in structure and microcirculation may cause an enzymic shift from one acinar zone to another (Eger, 1961; Sasse and Köhler, 1969).

Deductions made from experimental reversal of intrahepatic blood flow in dogs to disprove the dependence of the enzymic topography from the direction of intra-acinar blood flow are not valid. The "reversed liver" does not receive portal blood at all, and the venous (caval) blood flows in a direction opposing the arterial stream. Severe hepatic congestion and ascites occurred in the few surviving cases (Child, McDonough and

Des Rochers, 1959). In another preparation, the neck transplanted liver lobe (Sigel, Baldia, Brightman, Dunn and Price, 1968) is supplied by arterial blood only via the hepatic venous system; the blood does not pass through the arteriolar gates before reaching the sinusoids. In addition, biliary secretion was totally blocked through ligation of the bile ducts. One cannot accept such severely damaged preparations for the study of reversal of *normal* blood flow in the liver.

The zonal distribution of drug induced toxic hepatic lesions is due to the location of enzymes involved in the metabolism of the offending substance. Glucuronization of some drugs proceeds at a more efficient pace in zone 1, but the microsomes important for the biotransformation and detoxication of other drugs are more numerous in zone 3. Ohnhaus, Thorgeirsson, Davies and Breckenridge (1971) have reported that in rats hepatic blood flow increases by an average of 100% during the 4th day of enzymic induction with phenobarbitone; the increase in blood flow parallels the augmentation of the microsomal mass. Also, the rate of blood flow determines the quantity of substance brought to the cells to be metabolized. In a dog with an ischemic liver the detoxication of thiopental is delayed. If the ischemic liver is perfused *in situ* with additional blood from the dog's own aorta, the rate of detoxication of thiopental is increased (Rappaport, Hiraki, Rosenfeld, Cowan and Lang, 1956). These findings should remind us of the intimate connection between microcirculation and cellular function. Thus the histology based on the orientation of the parenchyma around the nutrient vessels and bile ductules becomes the chemomorphology of the acinus.

Figure 9 *Metabolic areas in the acini. This acinus can be considered as corresponding to the one on the right-hand side of the complex acinus (Fig. 5a). Specific enzymic activities indicate predominant metabolic functions in each of the microcirculatory zones of the acinus. The references in brackets indicate corresponding source. (a) = (Burstoue, 1959); (b) = (Eger, 1961; Klein, Widmer & Grossman, 1952); (c) = (Novikoff and Essner, 1960); (d & e) = Novikoff, Hausman and Podber, 1958); (f) = (Padykula and Herman, 1955); (g) = (Rutenberg and Seligman, 1955); (h) = (Schepers, 1961); (i) = (Wachstein, 1959); (j) = (Schumacher, 1957); (k) = (Greenberger, Cohen and Isselbacher, 1965); (l) = (Isselbacher and Jones, 1964); (m) = (Sasse, 1969; Sasse and Köhler, 1969); (n) = (Hayashi, 1964); (o) = (Albert, Orlowska, Orlowski and Szewczuk, 1964); (p) = (Mizutani, 1968); (q) = (Wachstein, Meisel and Falcon, 1961); (r) = (Johnson, 1967); (s) = (Balogh, 1966); (t) = (Reith, Schüler and Vogell, 1968); (u) = (Grisham, 1960); (v) = (Pette and Brandau, 1966); (w) = (Nolte and Pette, 1972); (x) = (Swick, Tollaksen, Nance and Thomson, 1970); (y) = (Shank, Morrison, Cheng, Karl and Schwartz, 1959). P.V. = portal vein; ThV = terminal hepatic venule; BD = bile ductule; hep. art. = hepatic arteriole; Z₁ = periportal area; Z₃ = perivenular area. (Rappaport, A. M., *Anatomic Consideration*, Chap. I, in L. Schiff ed., *Diseases of the Liver*, Lippincott, Philadelphia, 1974).*

It now remains to demonstrate that structure and microcirculation are seen *in vivo* the way they have been presented here. The film "Normal Microcirculation of the Mammalian Liver" (Rappaport, 1972)¹ shows the hepatic micro-structure and circulation as recorded during *in vivo* transillumination; it makes 6 points:

1. Vascular casts of the gross and microscopic afferent vessels are shown as the architectural framework of hepatic parenchyma. The technique of studying *in vivo* the microscopic vessels in the transilluminated liver is briefly demonstrated.

2. The hepatic microcirculation is organized in units around the end vessels of the hepatic artery and portal vein; each unit irrigates a corresponding liver acinus.

3. There are several patterns of distribution of blood into the sinusoids.

4. The confluence of the arterial and portal streams is shown at microscopic level; it is regulated through intermittent opening and closing of arteriolar sphincters. Flow is pulsatile in capillaries and sinusoids.

5. The hemodynamics resulting from this intermittent arteriolar activity are illustrated; the passage of arterial and portal blood under quite different pressures through the same sinusoidal network is explained.

6. The efferent hepatic venous stream is formed through confluence of the sinusoids with the terminal hepatic venules, and junction of the latter with each other.

Pathophysiology of the Liver Acini

In the liver acini the afferent vessels, particularly the arterioles, are the organizing principle of the hepatic parenchyma. The microcirculatory zones with their different tissue pO_2 and enzymic activity are bound to display different susceptibility to damage. The patterns of hepatic lesions (Rappaport and Hiraki, 1958) will be contingent upon the action on the parenchyma of the injurious agent as such or after its biotransformation. The site of the lesions will depend to a large degree on the circulatory pathways and will be either in zone 1, *i.e.*, "periportal" or at the microcirculatory periphery of the acinus, "perivenular", in zone 3 (Fig. 10).

The view of a hexagonal lobule as the smallest liver unit organized around a terminal hepatic venule ("central vein") offers to the pathologist only 2 or 3 landmarks for the orientation of lesions he observes. These are: "central vein", "portal periphery", and "midzone". The acinar concept, to the contrary, conveys a number of orienting lines and patterns in accordance with normal and impaired function of the hepatic acinus. There are six dynamic lines (Fig. 11A, 1-6) at the periphery of a regular hexagonal field by which adequate or deficient nutrients are delivered to the parenchyma and the produced bile is carried away. Therefore in liver biopsies it is easier to orient the tissue by studying first the parenchyma in the vicinity of the portal triads which in any slide are much more numerous than the ThV's. Pathological changes caused by noxious agents are brought to the parenchyma by the triadal pathways; the changes include biliary obstruction and ascending infections in the ductules and in the lymph vessels. The lesions may completely wipe out the parenchyma or may affect only cellular enzyme systems in certain zones of the acini of different anatomical order. Various patterns of pathologic lesions develop and reveal the shape of parts or of whole liver acini in the field. The distinction of these patterns is made easier by the fact that the tissue contained within one hexagon is not uniform. A hexagonal area is composed of unequal portions of several acini that hap-

¹ Copies available through the "Division of Instructional Media Services", Medical Sciences Building, Faculty of Medicine, University of Toronto, Toronto M5S 1A8, Ontario, Canada.

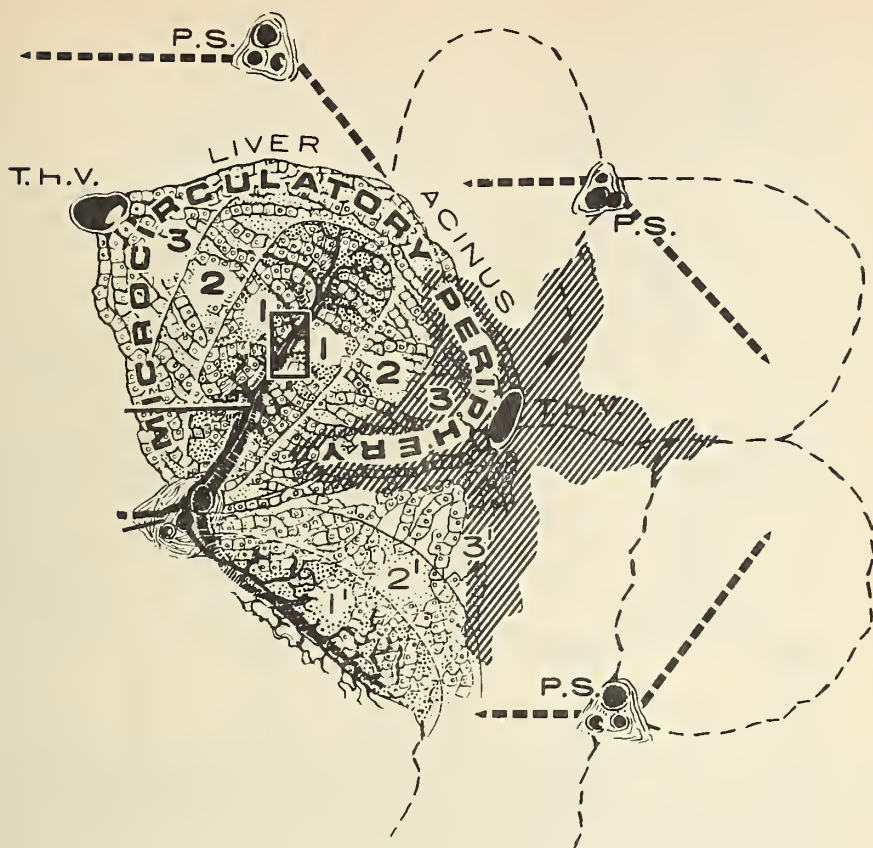


Figure 10 *Blood supply of the simple liver acinus, zonal arrangement of cells and the microcirculatory periphery. The acinus occupies adjacent sectors of neighboring hexagonal fields. Zones 1, 2 and 3 respectively represent areas supplied with blood of first, second, and third quality with regard to oxygen and nutrient contents. These zones center about the terminal afferent vascular branches, bile ductules, lymph vessels and nerves and extend into the triangular portal field from which these branches crop out. Zone 1¹, 2¹, 3¹ designate corresponding areas in a portion of an adjacent acinar unit. Zone 3 is the microcirculatory periphery of the acinus since its cells are as remote from their own afferent vessels as from those of the adjacent acini. The perivenular area is formed by the most peripheral portions of zone 3 of several adjacent acini. In progressive injury to zone 3 of these acini, the damaged area assumes the shape of a seastar (heavy crosshatching around a ThV in the center). 1, 2, 3 = microcirculatory zones; 1¹, 2¹, 3¹ = zones of neighboring acinus; - - - boundaries of acini; - - - afferent vessels of acini outlining the hexagons.*

opened to be exposed by the random histological section. Lesion 1 in Figure 11 B exemplifies a cut through the damaged tip of an acinus, part of the tissue contained within the realm of a hexagon. Lesion 2 is centered around the terminal afferent vessels that interdigitate with two ThV's. It shows the cross section of a liver acinus *entirely* diseased, and is seen e.g. in phosphorus poisoning or in fatty change due to severe starvation (Rappaport and Hiraki, 1958). Figure 11 C shows a lesion affecting only the most peripheral portions of zone 3 in complex acini. The damage is perivenular, but it disrupts already by stellate projections the contiguity between the complex acini; however, the simple acini are still in close contact with each other, and function together. The lesion can be caused by a fair

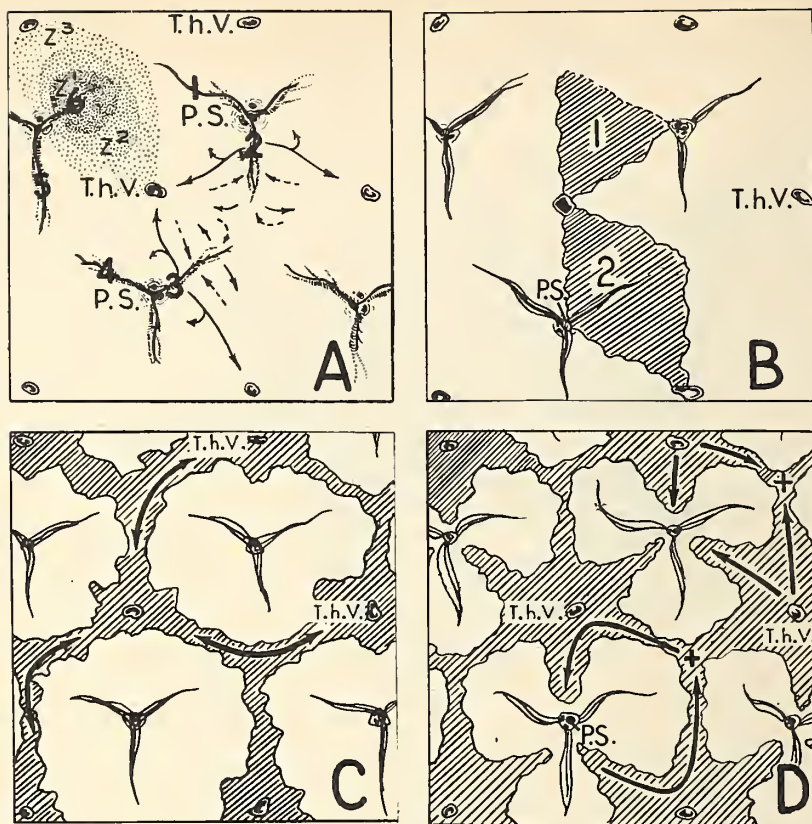


Figure 11 (A) Orienting lines in the parenchyma; patterns of hepatic injury. The six dynamic lines (1, 2, 3, 4, 5, 6) in an assumed regular hexagonal field indicate the pathways of blood supply (solid arrows) and bile drainage (broken arrows). They are the principal routes of invasion of the hepatic parenchyma. Z^1 , Z^2 , Z^3 = circulatory zones; PS = portal space; ThV = terminal hepatic venules.

(B) Hepatic lesions limited to single acini. Crosshatched area 1 = "paracentral" necrosis revealed by a tangential cut of a peripheral part of an acinus. 2 = transverse section of an entirely diseased acinus close to its axial vessels; the injury extends therefore into two sectors of neighboring hexagonal fields and reaches their ThV.

(C) Perivenular ("pericentral") necrosis results from mild damage to the parts of Z^3 most remote from the terminal afferent vessels of the complex acini contained within adjacent hexagonal fields. When the damage progresses perivenular necrosis becomes periacinar. The lesion around the ThV assumed a triangular shape. The triangularity is due to the extension of the injury that has crept along zone 3 and to the joining up with similar lesions around neighboring acini. Such linkage of ThV to ThV is also affected by fatty change or by fibrous strands. The uniform parenchyma has been reduced to disjointed clumps of tissue, the size of complex acini.

(D) The lesions developed in zones 3 and 2 have extended close to the portal spaces containing the preterminal afferent vessels. Small clumps of parenchyma have been isolated; they are surrounding the terminal afferent vessels as remnants of the simple acini. The arrows indicate the path of the advancing injury bridging the portal space (PS) to the ThV and breaking through the site where the capillarized axial structures of neighboring acini abut (X). The lesion also bridges ThV to ThV and the perivascular regions; the bands of injury curve and return to the initial portal fields. (From Rappaport and Hiraki, 1958.)

degree of ischemia (Rappaport, Macdonald and Borowy, 1953; Rappaport, Lotto and Loughed, 1954), by fatty change (Best, Hartroft and Lucas, 1949; Sellers and You, 1951), by carbon tetrachloride intoxication (Cameron and Karunaratne, 1936), in early dietary cirrhosis (Hartroft, 1953; Hoffbauer, 1959), in sclerosing hyaline necrosis (Edmondson, Peters, Frankel and Borowsky, 1967) of alcoholic cirrhosis (Lischner, Alexander and Galambos, 1971; Galambos, 1972) and in veno-occlusive disease due to senecio or fulvine poisoning (Bras and Hill, 1956). With progression of the lesions around the complex acini the normally invisible peripheries of the single acini become conspicuous. This happens most commonly in congestive heart failure (Fig. 15) necrotic, fatty or fibrous damage. The bands of injury in a perivenular seastar-like pattern (ThV right upper corner, Fig. 11 D) have reached on one side the portal triad from which the terminal afferent vessels of the affected acinus have branched out. The bands have also extended to the site where the tips of the nutrient vessels of several neighbouring acini have dwindled down to capillaries (Fig. 11 D, +), the so-called "nodal" point (Mall, 1906). From this point the band of damage curves and returns to the original portal triad. The grave prognostic significance of such "bridging" between two ThV's in neighbouring hexagonal fields, or of the ThV's to the portal triads has been expounded by Boyer and Klatzkin in 1970, in a fine clinical study of subacute hepatitis. However, the reason for the continued deterioration of liver function in these patients is now evident from the

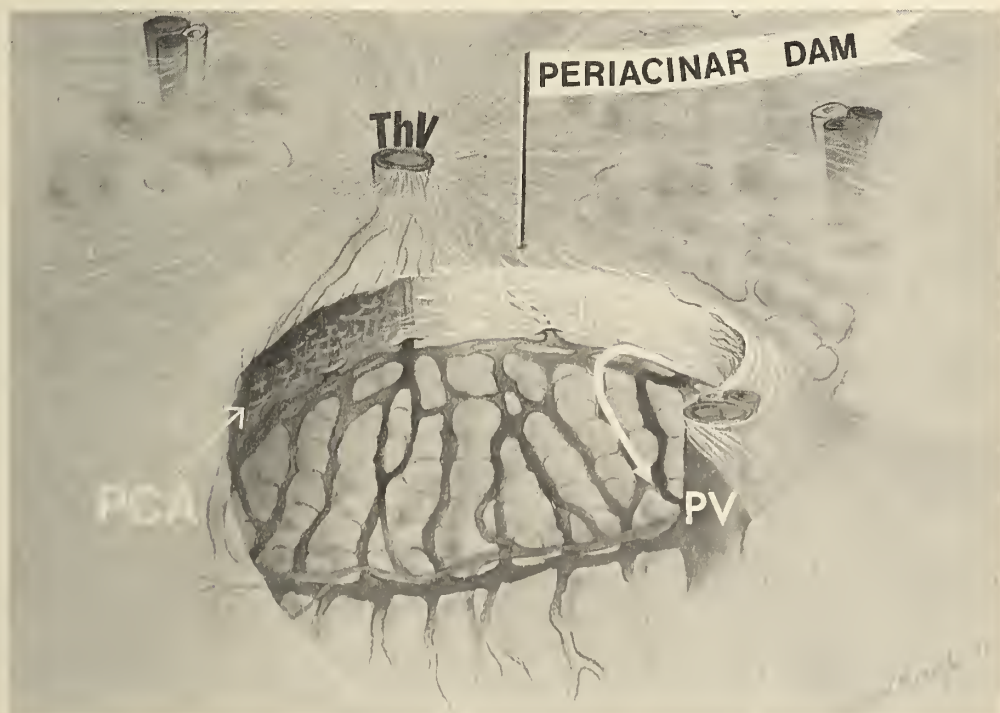


Figure 12 Perivenular fibrous dam causing postsinusoidal portal hypertension. The number of sinusoidal openings into the ThV has been greatly reduced through fibrous obliteration. Sinusoidal blood under arteriolar pressure bypasses the obstacles via a porta-caval shunt (PCA) that collects the blood from venules, part of a venous plexus that has formed around the nodules. When the arteriolar activity supervenes in the microcirculation some arterial blood may bypass the sinusoids and empty directly into the portal venules (PV) thereby reversing the direction of blood flow in them.

pathophysiology of the hepatic microcirculation. The circulatory intercommunication between the individual acini is compromised; each of them is relying only on the capacity of its own cells to resist. It is not unlike an army being split up by an invading enemy into small encircled yet still resisting groups with little chance of forces reuniting.

The surviving parts of the acini (zone 1 and 2) remain thus enclaved in a layer of damaged tissue, which in the healing process may become replaced by a densely woven scar. This is most frequently the case in alcoholic cirrhosis. In the sclerosed acini the scar is acting as a periportal dam (Fig. 12) to the outflow of blood into the systemic veins. One may get the impression that our liver tries hard to stave off the repeated flooding of the systemic veins with alcohol by the chronic imbibitor. Any dam, however, raises the level upstream, and so the postsinusoidal barriers raise the pressure level in the portal stream. Portal hypertension will ensue. A closer view of the histogenesis of the various forms of cirrhosis is facilitated by Figure 13. Periportal rims, islets or clumps of tissue surviving in ischemic necrosis, in CCl_4 poisoning, in hepatitis, in the fatty liver of the alcoholic and in experimental cirrhosis (Best, Hartroft, Lucas and Ridout, 1955) are found to be oriented around the terminal (Fig. 13 A, 1) and preterminal (Fig. 13A, 2) afferent axial vessels and bile ducts. Conversely the ThV ("central veins") have become isolated, "divorced," from the portal afferents by bands of damaged tissue. The same figure 13 A, 2 depicts a complex acinus, with its central trident of surviving tissue around the preterminal vessels and the vaulted areas of damage at its periphery.

A band of injured tissue connecting several hepatic venules to each other and to portal fields may arch like the broken line in Figure 13 A over many hexagonal fields to surround an area of tissue that represents the cross section of an acinar agglomerate (Fig. 13A, 3). Within this area the hepatic parenchyma may be undamaged because the pathological change was not yet severe enough to injure the peripheries of the single acini. Such relationship is not always visible in a single section but can be disclosed on serial sectioning.

When the damaged tissue is replaced by fibrous bands they will crisscross the parenchyma annihilating completely any regular design of interdigitation between afferent and efferent vessels. Instead of polygonal figures, a nodular pattern becomes conspicuous in which parenchymal clumps of various sizes are centred around preterminal (Fig. 13B, 2) or terminal (Fig. 13B, 1) afferent vessels. This "pseudolobulation" represents in fact the regenerated parenchymal core of the damaged acini, the "pseudolobules" differing in size and shape according to their original anatomical order. The ThV at the periphery of the nodules, the hepatic venules, have not changed the position they occupy normally, *i.e.* at the *periphery* of the acinar clumps.

The common pathology as occurring in the simple liver acini is illustrated in Figure 14; the diagrammatic acinus corresponds in its orientation to the right hand acinus seen in Figure 5. The topography of the lesions can be described as either "periportal" or "periportal" (perivenular). "Periportal" is identical with zone 1 of the acinus, *it can by no means be considered a "peripheral" area, since it is the most active circulatory region in the parenchyma*. Here the arterial and portal streams merge and with their junction the perfusion of the parenchyma begins. Zone 2 is a strip of parenchyma representing the gradual transition of the tissue pO_2 from zone 1 to zone 3. As the arteriolar activity increases, zone 1 will increase in width at the expense of zone 2. Conversely, in anoxia or circulatory failure zone 3 may increase in width at the expense of zone 2. Zone 3, identical with the microcirculatory periphery of the acinus (Fig. 14), is more prone to damage by circulatory insufficiency. When fibrosis linking ThV to TPV and new formation of vessels develop intrahepatic portacaval shunting will occur (Fig. 14).

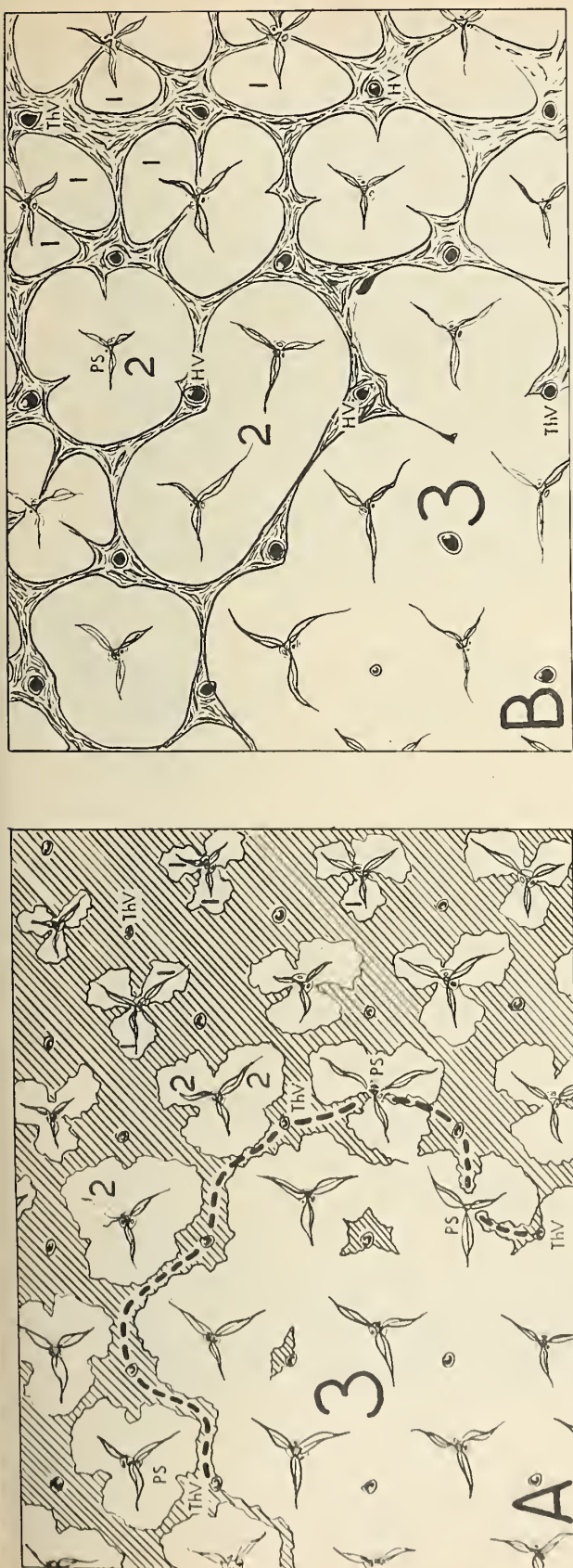


Figure 13 (A) Hepatic damage affecting simple acini (1), complex acini (2) and an acinar agglomerate (3). The lesion is most severe in the right half of the diagram, where the simple acini have lost most of their parenchyma and have been reduced to small 'periportal' rims of tissue (1) around the nutrient vessels. The injury is less in the center of the figure where complex acini (2) have survived. However, they are already separated from each other and from the bulk of a well preserved acinar agglomerate by strands of damaged and scarring tissue (broken line) linking the portal spaces (PS) to terminal hepatic venules (ThV) situated at the microcirculatory periphery of this acinar agglomerate.

(B) Regeneration of the acinar remnants. Regeneration and hyperplasia, starting from the surviving remnants of the acini have created a nodular pattern of the hepatic parenchyma. The monoacinar nodules (1) developed from the remainder of simple acini can easily be distinguished from small nodules (2) formed out of surviving remnants of complex acini. The large node (3) is the result of hyperplasia in a preserved acinar agglomerate. Note that the hepatic veins are in their normal position, i.e. at the periphery of the acini. (From Kappaport and Itraki, 1958.)

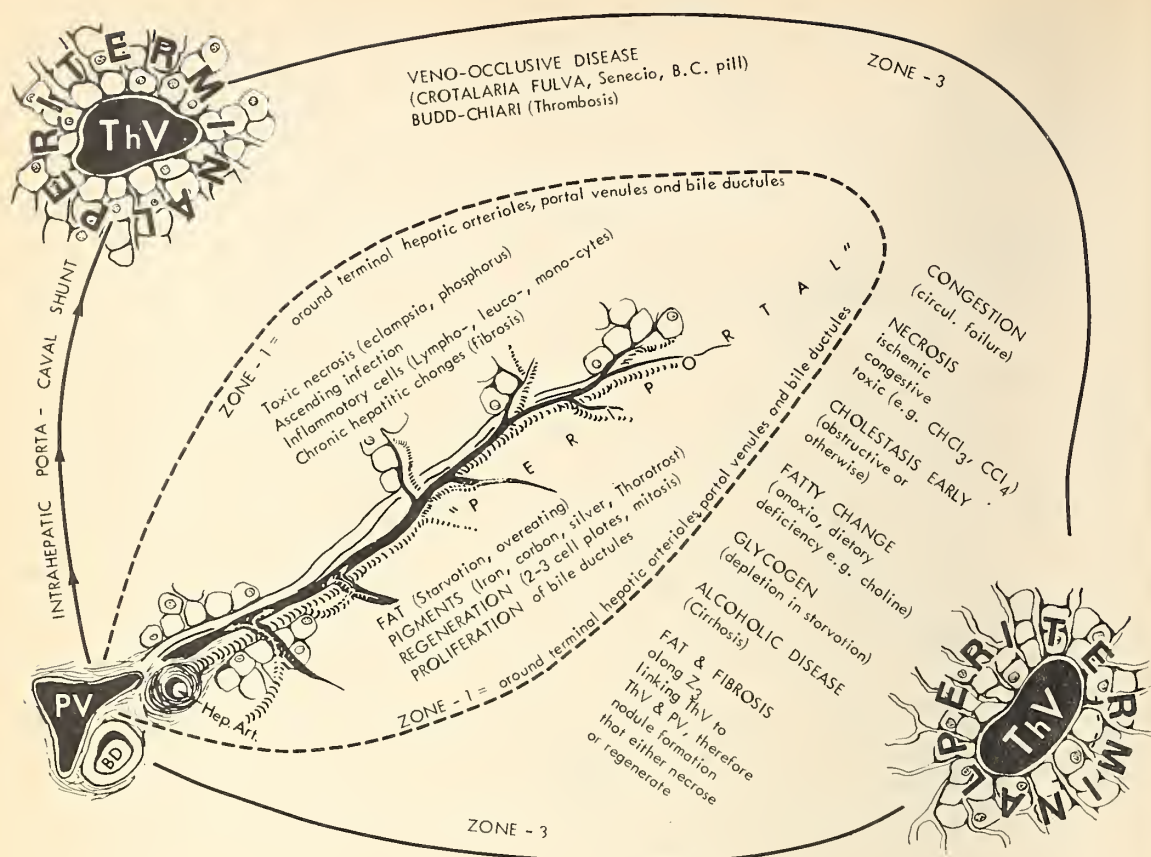


Figure 14 Diagram summarizing the localization of common lesions in the liver acinus. Only zone 1 and zone 3 are shown as there is no lesion characteristically situated on the borderline (i.e. zone 2) of these areas. Zone 1 is correctly called 'periportal', it can by no means be termed 'peripheral' as here, at the junction of the arterial and portal streams, is the hub of the microcirculation. 'Periterminal' = region around the terminal hepatic venule (ThV), it is therefore synonymous with 'perivenular'. The area is composed of portions of zone 3 of acini adjacent to ThV.

SUMMARY

The architecture of the liver has been described in connection with the microcirculation as it presents itself *in vivo*. The structural unit, the simple liver acinus, contains the microcirculatory hepatic unit; both are organized around the terminal hepatic arteriole and terminal portal venule. All arterioles empty exclusively into the periportal area and with their fully oxygenated blood create a pO_2 gradient decreasing towards the perivenular area, i.e. around the ThV, situated at the microcirculatory periphery of the acini. The pO_2 difference in the parenchyma close to and remote from the afferent vessels creates diverse microenvironments suitable for specific enzymic activities in the respective areas. Thus the hepatic cells display metabolic heterogeneity and different susceptibility to damage.

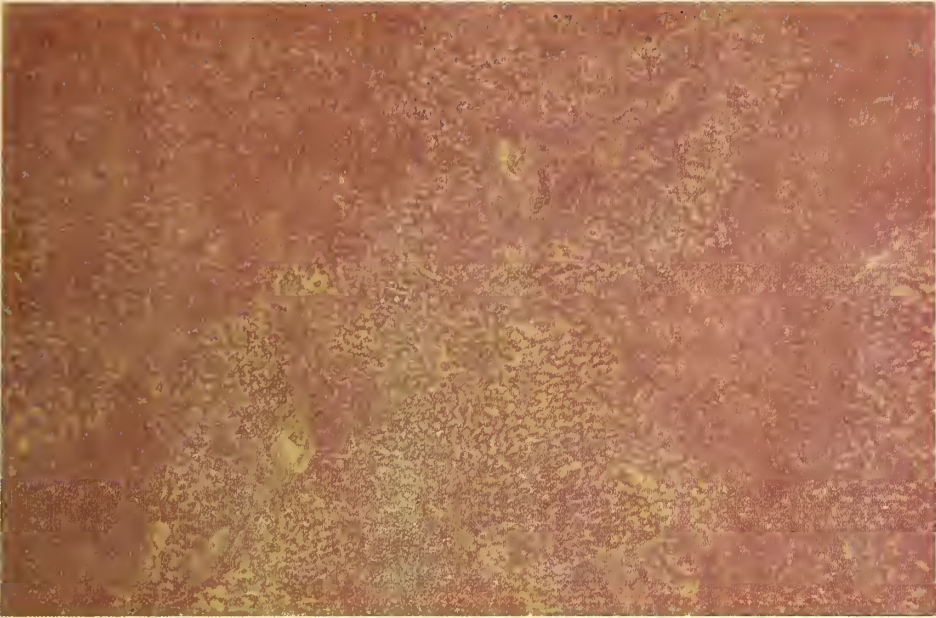


Figure 15 *Liver in congestive heart failure. The microcirculatory periphery of a complex acinus is outlined by broad bands of erythrocytes stagnating in dilated portions of the sinusoids, that are most distant from the afferent vessels. These bands ('Staungsstrassen') link ThV to ThV. (Courtesy Dr. Ritchie, Toronto General Hospital, Toronto).*

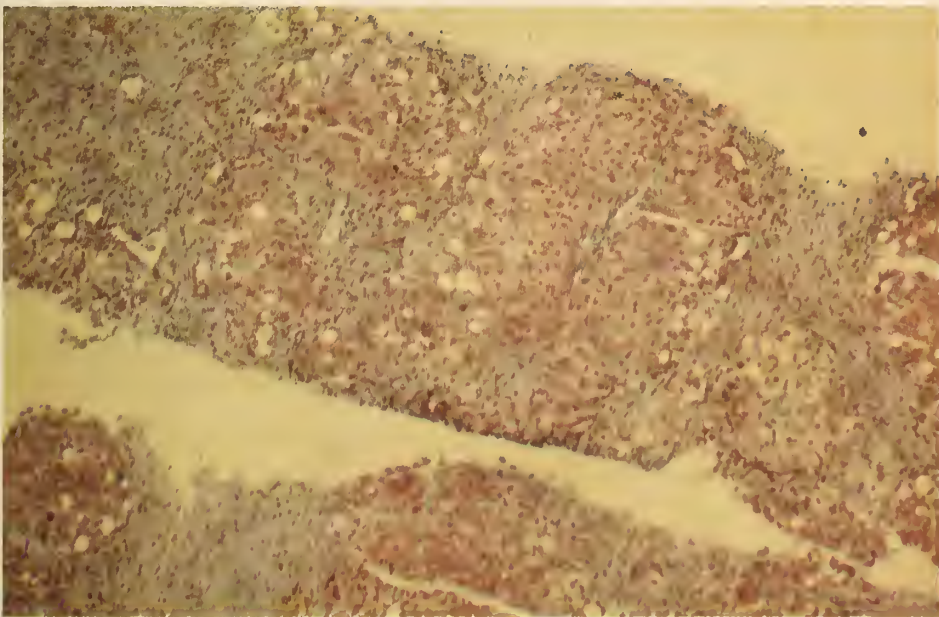


Figure 16 *Liver biopsy from a patient with cirrhosis. Zone 3 of several acini is marked by fibrous bands, a result of toxic hepatitis. The 'lobule splitting cirrhosis' reveals the acinar remnants as nodules centered around their triads. Note that there are more portal fields than ThV's in a needle biopsy specimen. (Courtesy Dr. Medlin, Toronto Western Hospital.)*

The orientation in hepatic histopathology has been presented based on the pathophysiology of the liver acini. It provides better understanding and a unifying view of liver pathology. Conversely, the description of hepatic damage by making its distance from the terminal hepatic venules ("central veins") as the prime point of explanation leads to arbitrary, endless grouping and subgrouping of pathologic entities. Such textbook orientation is remote from taking into account the factor vital to the liver as to any other organ — the microcirculation.

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The Hepatic Circulation and Portal Hypertension in Alcoholic Cirrhosis

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The hemodynamic changes associated with alcoholic cirrhosis are dominated by the hypertension created in the portal venous system. The major obstruction to hepatic venous outflow is presumed to be at a sinusoidal and post sinusoidal level. The small hepatic veins, as well as the sinusoids, are compressed by the regenerating nodules which are generally accepted to be more responsible for vascular obstruction and portal hypertension than is fibrosis (Kelty, Baggenstoss and Butt, 1950). However, in acute alcoholic hepatitis, without cirrhosis, the hyaline necrosis and collagen deposition in the central portion of the liver nodule may be associated with marked elevation of the portal pressure, prior to the development of nodules (Reynolds, Hidemura, Michel and Peters, 1969). In the regenerating nodule, shunts from the portal venules to the excentrically displaced central veins may exist, presumably through the link of persistent sinusoids in the septa (Popper, Elias and Petty, 1952). Anastomosis, between distal branches of the hepatic artery, of portal veins and of hepatic veins, also increases with the progression of liver disease (Mitra, 1966). Previously, it was generally accepted that the regenerating nodules, deprived of portal blood supply, were vascularized mostly by hepatic arterial branches. However, recent studies indicate that in rats and humans with cirrhosis, the portal vein remains the chief source of blood supply to the nodules and that the hepatic arterial inflow varies (Chenderovitch, 1961; Baldus and Hoffbauer, 1963; Mitra, 1966). Arterial capillaries, increased in number, terminate mainly in the broad bands of connective tissue and in the proliferated bile ducts (Mitra, 1966).

Portal hypertension and the development of a collateral circulation are major hemodynamic problems following pathological changes associated with advanced alcoholic cirrhosis of the liver. Hemorrhage from esophageal and/or gastric varices is still the main cause of death, either from exsanguination or from hepatic failure (Sherlock, 1964).

mainly in alcoholic patients who continue to drink (Powell and Klatskin, 1968). An average mortality rate of 55% of all cirrhotic patients has been reported one year after the initial hemorrhage (Garceau, Chalmers and the Boston Inter-hospital Liver Group, 1963; Sherlock, 1964). Portal decompression following portosystemic anastomosis has proven to be efficacious in preventing recurrent variceal bleeding, if the shunt remains patent (Linton, Ellis and Geary, 1961). However, the longterm benefits of surgical procedures are not obvious, since one of the consequences of standard portacaval shunts is the complete loss of portal blood flow: reduced incidence of bleeding varices is associated with a higher mortality rate, due to early or progressive hepatic failure, or other undesirable metabolic sequelae, such as encephalopathy (Read, Laidlaw and Sherlock, 1961); prospective controlled studies of prophylactic shunts indicate that this procedure does not enhance survival, and indeed, probably diminishes it (Jackson, Perrin, Smith, Dagradi and Nadal, 1968; Resnick, Chalmers, Ishihara, Garceau, Callow, Schimmel and O'Hara, 1969; Conn and Lindenmuth, 1965 and 1969); a more favorable effect is reported by Mikkelsen (1974), with an improved five year survival, following therapeutic shunt in patients who bled from varices, at least once.

Ability to predict which patient will bleed from esophageal and/or gastric varices, which patient will tolerate surgery better, as well as the selection of the surgical procedure most appropriate for each individual case, have incited many investigations in this field. All the splanchnic hemodynamic investigations discussed below have proven to be helpful in the diagnosis and physiological and/or clinical evaluation of alcoholic cirrhotic patients with portal hypertension. So far, most of these investigations, generally used in selecting patients for portal systemic shunts, cannot be correlated with the post-operative course. Therefore, their usefulness has been questioned (Reynolds, 1974a). However, no existing studies were performed adding, to other hemodynamic parameters, the pre-operative measurement of the portal fractions of hepatic blood flow. This measurement has never been satisfactorily evaluated in conscious man, because of the dual blood supply to the liver and the relative inaccessibility of the portal vein.

DIAGNOSTIC STUDY OF THE PORTAL VENOUS SYSTEM

In alcoholic cirrhosis, the starting point of such studies is not only linked with the presence of esophageal and/or gastric varices, but also with an accurate diagnosis of their rupture during eventual bleeding episodes. Emergency endoscopy, when performed during active bleeding, is the choice procedure in the differential diagnosis of upper gastrointestinal bleeding (Palmer, 1970). Esophago-gastrosocopy allows visualization of bleeding varices and can, in most instances, discriminate between alcoholic esophagitis or gastritis, Mallory-Weiss tears and gastroduodenal ulcers, to enumerate other frequent bleeding lesions in alcoholics (McCray, Martin, Amir-Ahmadi, Shzahan and Zamchek, 1969; Conn, 1970; Khudadoost and Glass, 1972). At Hôpital Saint-Luc, from January 1970 to July 1974, 366 early fiber optic esophago-gastrosopies were performed because of upper gastro-intestinal bleeding (F. Martin and A. Farley, personal communication). There were 84 bleeding episodes in 77 patients with alcoholic cirrhosis and esophageal and/or gastric varices: in 36 bleeding episodes, ruptured varices were the sole source of hemorrhage; in 43 cases, at least one other source of hemorrhage was found, associated with non-bleeding varices, such as esophagitis or gastritis (19 cases), Mallory-Weiss syndrome (6 cases) and gastroduodenal ulcers (14 cases). In the last 5 cases, no definite source of bleeding could be identified. The varices were graded according to their diameter at endoscopy: the

diameter was greater or equal to 3-4 mm in all cases where a ruptured varix was identified; however, in cases where another source was found, the diameter varied from 1 mm to more than 5 mm. The variceal size found at endoscopy usually correlates with the degree of portal hypertension (Willoughby, David, Smith, Fruin and Baker, 1964; Aoun, Guillaume, Martin, Farley, Mheir, Légaré, Lavoie, and Viallet, 1971; Dagradi, 1972), but, although only large varices have been reported bleeding (Dagradi, 1973), the variceal size itself is not diagnostic of bleeding in patients with severe portal hypertension. These findings demonstrate that, in alcoholic cirrhotic patients, ruptured varices should not be incriminated as the source of upper gastrointestinal bleeding, only because of their presence or their size.

Aside from diagnosing the hemorrhagic site, a good evaluation of the degree of portal hypertension is necessary in the management of patients with alcoholic cirrhosis. Direct measurements of the mesenteric or portal vein pressure have first been taken at surgery, during laparotomy, through direct puncture of vessels (Taylor, 1954; Moreno, Burchell, Rousselot, Panke, Slafsky and Burke, 1967). Several methods, avoiding anesthesia and surgery, have been proposed for indirect estimation of the portal pressure. Currently, splenic pulp pressure (Atkinson and Sherlock, 1954; Foster, Conkle, Crane and Burko, 1974), wedged hepatic venous pressure (Myers and Taylor, 1951) and intrahepatic tissue pressure (Vennes, 1970) are the most commonly used measurements.

Percutaneous intrasplenic puncture provides a pressure measurement of the splenic pulp which communicates freely with the intrasplenic venous radicles of portal tributaries (Atkinson and Sherlock, 1954). Therefore, intrasplenic pressure is in close relation with the portal venous pressure or presinusoidal pressure. Intrasplenic pressure measurements are usually recorded only with an external zero reference (5 cm below sternal angle with the patient supine). A good correlation has been found in patients with and without portal hypertension, between the splenic and wedged hepatic venous pressure, or portal pressure at surgery (Atkinson and Sherlock, 1954). However, in a recent study, Foster *et al.* (1974) compared the pre-operative splenic pressure and the per-operative portal pressure in 31 patients with portal hypertension: in only 12 patients the difference between both pressures was less than 5 cm H₂O, and in the 19 other patients, a wide difference was noted. Although pressures recorded in the spleen have been found to differ very little in different parts of the organ (Atkinson and Sherlock, 1954; Semin, 1958), high intrasplenic pressures were occasionally recorded, probably due to the proximity of the needle tips to a branch of the splenic artery (Arner and Fernström, 1960).

Percutaneous intrasplenic puncture also allows a potential combination with spleno-portal venography which is a commonly used technique in the evaluation of anatomic and hemodynamic changes associated with portal hypertension (Rousselot and Burchell, 1969). The risk of splenic hemorrhage, although minimal, has defined splenic puncture almost as a pre-operative measure. In 10 to 15% of procedures, the impossibility of discriminating between an occluded and a patent, but non visualized portal vein (pseudothrombosis), has also remained one of its main limitations in the management of patients with cirrhosis and portal hypertension (Burchell, Moreno, Panke and Rousselot, 1965). Ascites, thrombocytopenia and/or prothrombin deficiency are contra-indications, because of an increased risk of serious bleeding from the splenic puncture site. In cases of contra-indication, after splenectomy and also when the portal vein is not visualized by splenoportography, an arteriography, via the celiac or mesenteric artery, can be of great value in showing the splenic or mesenteric and portal veins on venous return (Rigler, Olfelt and Krumbach, 1953; Hepp, Hernandez, Moreau and Bismuth, 1968; Conn and Ramsby, 1973).

Hepatic vein catheterization is relatively simple and safe for the measurement of hepatic vein pressure in the free and wedged positions (FHVP and WHVP). In cirrhosis of the liver, a close relationship between WHVP and operative portal pressure was found by several authors (Myers and Taylor, 1951; Paton, Reynolds and Sherlock, 1953) and this method has been widely used for an indirect measurement of sinusoidal and/or post sinusoidal pressure in portal hypertension. With the development of umbilico-portal cannulation, a direct approach in the portal vein allowed a comparison between WHVP and free portal venous pressure (FPVP) in conscious patients and showed nearly identical values in cirrhosis of the liver (Viallet, Joly, Marleau and Lavoie, 1970a). Figure 1 illustrates the results obtained in 50 alcoholic cirrhotic patients, evaluated at Hôpital Saint-Luc. With the hepatic vein catheterization, an internal zero reference is available: this can be either pressure recorded in the inferior vena cava or in one hepatic vein in the free position (Reynolds and Redeker, 1965); both are preferred to central venous pressure or right atrial pressure as an internal baseline in cirrhotic patients, because of an eventual abdomino-thoracic pressure gradient, particularly in presence of ascites (Iwatsuki and Reynolds, 1973). Therefore, the porto-hepatic gradient, defined as the WHVP (or FPVP) less the FHVP, is independent of systemic circulatory conditions and intra-abdominal pressure. This parameter is considered to be the most reliable parameter in the evaluation of portal hypertension in cirrhotic patients. The range of porto-hepatic gradient in alcoholic cirrhosis was reported to vary between 5 and 26 mm Hg, while in normal subjects, this range probably does not exceed 5 mm Hg (Lundberg and Strandell, 1973; Bynum, Hanley and Cole 1973); however, in some cases of alcoholic cirrhosis, proven by liver

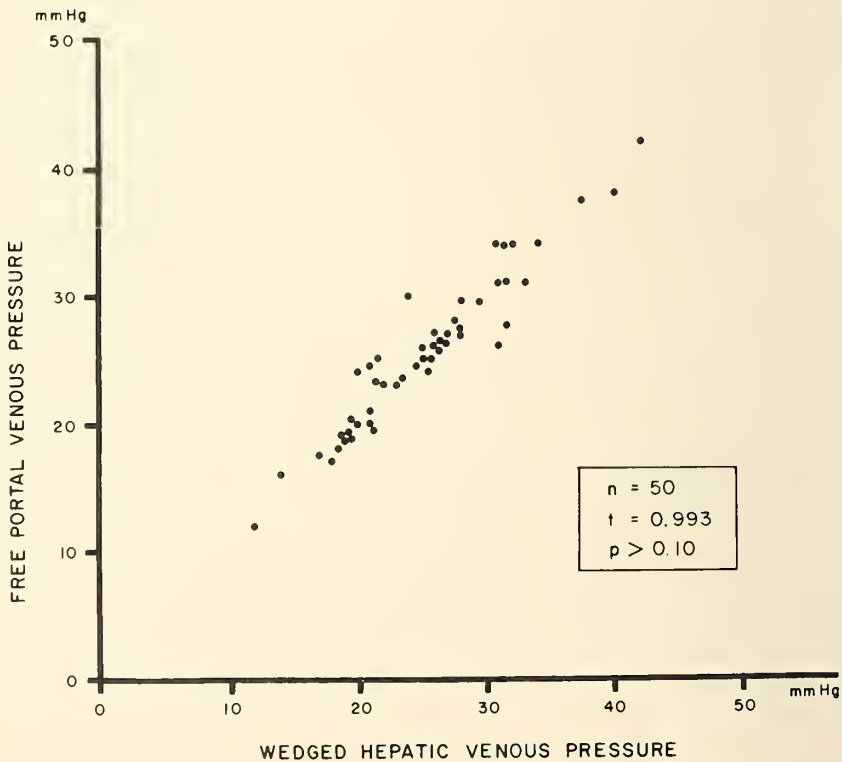


Figure 1. Comparison of free portal venous pressure and wedged hepatic venous pressure recorded simultaneously in 50 alcoholic cirrhotics.

biopsy, no portal hypertension could be found (Viallet, Bernier, Marleau and Lavoie, 1971). The retrohepatic compression of the vena cava by the liver can also be evaluated with the catheter positioned in the abdominal vena cava before portacaval shunts (Mullane and Gliedman, 1970).

In alcoholic patients, portal pressure has been shown to decrease spontaneously with clinical improvement, after prolonged abstinence (Leevy, Zinke, Baber and Chcy, 1958; Reynolds, Geller, Kuzma, and Redeker, 1960). Therefore, alcoholic hepatitis should not be present at the time of pre-operative hemodynamic evaluation, when portacaval shunting is considered. WHVP decreases after portacaval shunt, more so after side-to-side than after end-to-side shunts (Reynolds, 1970). Since no correlation has been found between the pre-operative WHVP and post-operative course (Reynolds, 1970), the main usefulness of the WHVP is in the assessment of patients presenting with alcoholic cirrhosis and upper gastrointestinal bleeding: a WHVP less than 10 mm Hg would suggest that esophageal and/or gastric varices are not the source of bleeding (Reynolds, 1974b). Hepatic vein catheterization can also be used for wedged hepatic venography (Celis, Villalobos, Del Castillo and Flores-Espinosa, 1955) with visualization of reversal portal blood flow in some cirrhotic patients, either spontaneously or following portacaval shunts (Warren, Fomon, Viamonte, Martinez and Kalser, 1968; Reuter and Orloff, 1974).

Estimation of Hepatic Blood Flow

Estimation of hepatic blood flow based on hepatic extraction (or hepatic clearance) of bromsulphalein was first described by Bradley *et al.* in 1945, utilizing a constant infusion of the dye. This technique can be applied to conscious man. Following continuous infusion of bromsulphalein or, more recently, of indocyanine green (Caesar, Shaldon, Chiandussi, Guevara, and Sherlock, 1961), the dye concentration is determined simultaneously in hepatic venous blood and in arterial blood. Since the liver is the major organ concerned with dye extraction, the concentration in the hepatic artery is identical to that of portal vein (Figure 2) (Viallet, A., unpublished data), and the Fick formula may be used in the estimation of hepatic blood flow.

Other clearance methods have been described, using peripheral plasma disappearance after a single injection of a substance selectively removed during one passage through the liver, either by hepatocytes (Banaszak, Stekicz, Grace and Smith, 1960) or Kupffer cells (Dobson and Jones, 1952; Halpern, Biozzi, Pequignot, Delaloye, Stiffel and Mouton, 1959; Benhamou, Nicollo, Girond, Tricot, Léger, and Fauvert 1961; Shaldon, Chiandussi, Guevara, Caesar and Sherlock, 1961). Flows measured are underestimated because of incomplete hepatic extraction, and are therefore called minimal hepatic blood flows. Estimation of hepatic blood flow necessitates correction using the extraction rate, which can be calculated only by simultaneously performing hepatic venous catheterization, because of a decreased hepatic extraction (Halpern *et al.*, 1959; Shaldon *et al.*, 1961) and an increased extra-hepatic uptake of colloids (Millette, Chartrand, Lavoie and Viallet, 1973a) in cirrhosis of the liver.

All these methods are less reliable in accurate hepatic blood flow measurements in alcoholic cirrhosis, as the hepatic extraction decreases considerably with the severity of the liver disease (Cohn, Khatri, Groszman and Kotelanski, 1972). Moreover, regional variation in the hepatic extraction has been described in advanced cirrhosis of the liver (Bradley, Ingelfinger, Bradley and Curry, 1945; Strandell, Erwald, Landbergh and Wiechel, 1973).

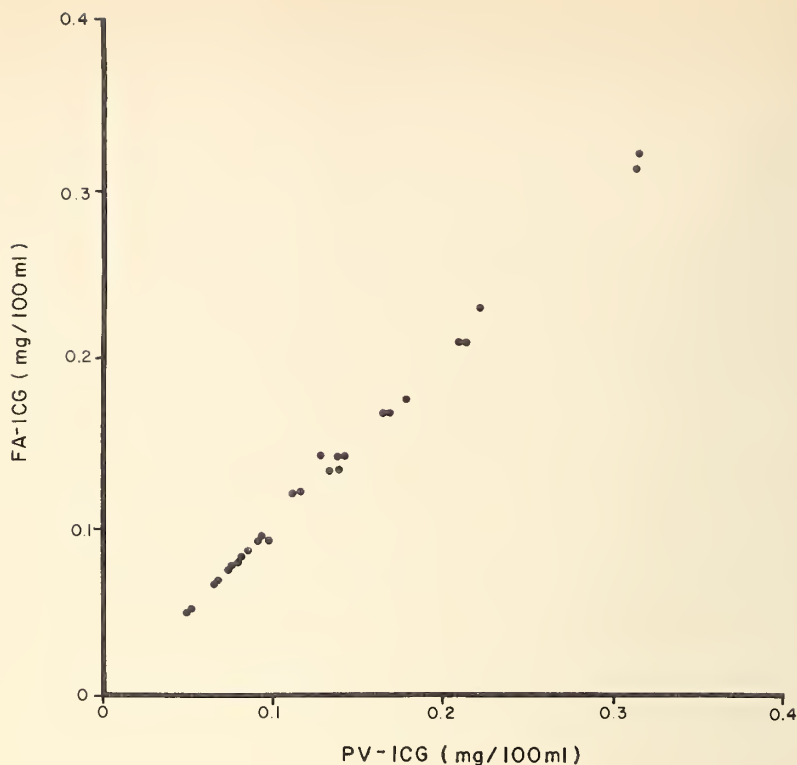


Figure 2. Comparison of the mean indocyanine green concentration measured simultaneously in the portal vein (PV-ICG) and femoral artery (FA-ICG) during a continuous infusion in 27 cirrhotics.

Indicator dilution methods, not dependent on hepatic function and therefore, more accurate in liver disease, were used in the estimation of hepatic blood flow after injection of an indicator into the spleen (Reichman, Davis, Storaasli and Gorli, 1958). Recently, this method was applied in normal and cirrhotic subjects, using injection of a labelled indicator into the hepatic artery and sampling from hepatic veins (Cohn *et al.*, 1972). In normal subjects, no difference was found between flow values estimated by this method and by the indocyanine green disappearance method. In certain cases, nearly identical flows were calculated simultaneously from two hepatic veins. These findings, if confirmed in cirrhosis, suggest that adequate mixing of the indicator is achieved within the hepatic circulation, after injection of the indicator into the hepatic artery.

This method has been applied to cirrhotic patients in the evaluation of porto-systemic shunts from the splenic and mesenteric beds, by comparing curves obtained from hepatic veins after injecting into the hepatic artery and splenic or mesenteric arteries (Groszmann, Kotelanski, Cohn and Khatri, 1972).

Estimation of hepatic blood flow with methods using hepatic arterial injection would be advantageous, as these are independent of hepatic function and do not necessitate splenic (or portal) injection. However, selective hepatic arterial catheterization is required distal to any extra-hepatic collateral, such as the gastroduodenal artery, and results are unreliable when arterial supply to the liver is derived from more than one hepatic artery, as it frequently occurs in man (Suzuki, Nakayasu, Kaware, Takeda, and Honjo, 1971).

As a general concept, the evolution of alcoholic cirrhosis establishes a vascular resistance in the liver with the development of a collateral circulation, which should gradually reduce hepatic blood flow. However, in alcoholic cirrhosis, with or without alcoholic hepatitis, a wide range of flows has been reported (Bradley *et al.*, 1948; Reynolds, 1970; Cohn *et al.*, 1972), with values lower as well as higher than in normal subjects (1,000 to 2,000 ml/min, mean 1,500 ml/min). No correlation has been found, either with the degree of portal hypertension (Joly, Marleau, Lavoie, Bernier, Joubert and Viallet, 1969) (Figure 3), or with the degree of hepatic failure (Lebrec, Sicot and Benhamou, 1973).

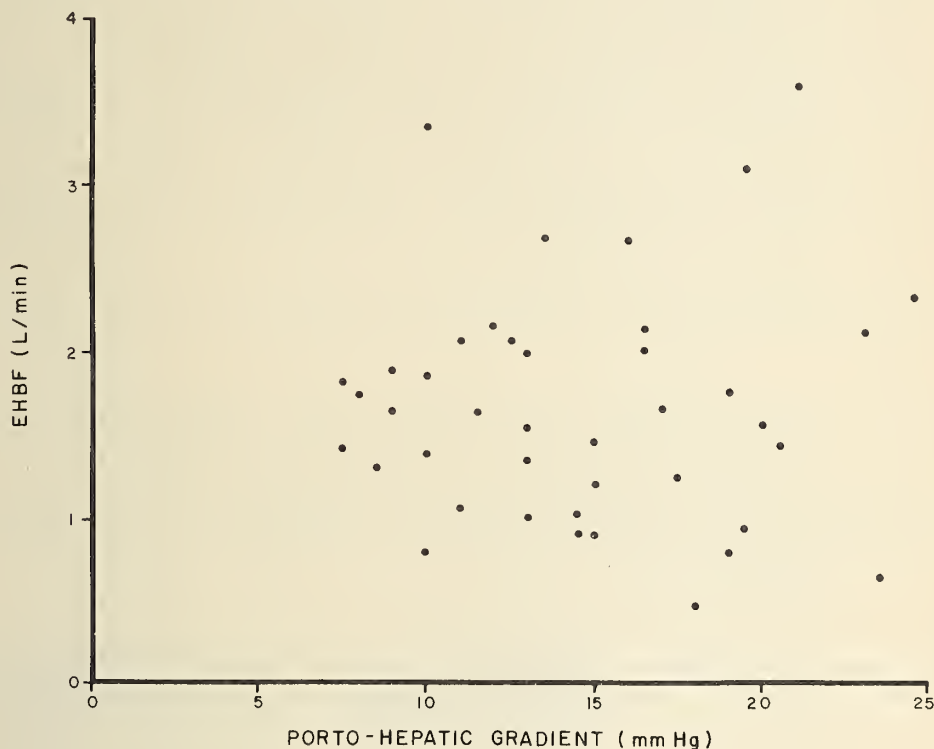


Figure 3. Comparison of estimated hepatic blood flow (EHBF) and portohepatic gradient measured simultaneously in 41 cirrhotics.

Even when total flow is unreduced, the fractional or effective hepatic blood flow to the hepatocytes may be lessened, because of intrahepatic porto-hepatic shunts (Popper *et al.*, 1952; Mitra, 1964) or because of the development of a basal membrane along the sinusoidal endothelium (Schaffner and Popper, 1963). Estimation of this functional hepatic blood flow was proposed, using the hepatic extraction efficiency of colloidal heat-denatured human serum albumin labelled with I^{131} (Halpern *et al.*, 1959; Shaldon *et al.*, 1961). In normal animals and subjects, the colloid is almost completely removed (more than 90%) by Kupffer cells during one passage through the liver, although in cirrhotic patients, reported values varied between 64 and 90% (Halpern *et al.*, 1959; Shaldon *et al.*, 1961). However, the possible increased extra-hepatic uptake of the colloid was not evaluated, a known phenomenon in liver cirrhosis, when using other colloids (Millette *et al.*, 1973a).

Until the development of umbilico-portal cannulation, few non-surgical methods were proposed for a separate measurement of hepatic arterial and portal blood flows (Ueda, Unuma, Iio and Kameda, 1962; Nakamura, Nakamura, Aikawa, Kera and Sasaki, 1971; Marleau, Hoanca, Pointard and Benhamou, 1971), but most of these studies were not based on an experimental model. Hepatic arterial and portal blood flow values reported were those taken during laparotomy, prior to portosystemic shunts, using electromagnetic flowmeters (Price, Voorhees and Britton, 1967; Burchell, Moreno, Panke and Nealon, 1974). In one of the largest series (Burchell *et al.*, 1974), a wide range of flows recorded, varied from 70 to 1,080 ml/min for the portal vein and from 130 to 860 ml/min for the hepatic artery; the ratio between the hepatic artery to the portal vein flows varied from 0.23 to 4.71; in a few cases, a stagnant or reverse portal flow was observed. In this report, Burchell *et al.* found no correlation between the portal blood flow and the "maximum perfusion pressure" (or the difference between pressures on the hepatic and splanchnic side of a clamp occluding the portal vein) generally used by surgeons as an evaluation of portal vein inflow (McDermott, 1972). Moreover, Burchell *et al.* could not correlate the portal blood flow or the "maximum perfusion pressure" with the post-operative course. Direct measurement at surgery allows an immediate evaluation of the hepatic artery response after portal vein clamping: considerable variations in the changes of the hepatic arterial flow, varying between 0 and more than 100%, from one patient to another were compiled by Reynolds (1974b). Marked increase in the hepatic arterial flow might be associated with good clinical results after surgery (Burchell, Rousselot and Panke, 1968).

As to the usefulness of hemodynamic data provided by per-operative flowmeter measurements, it should be considered that this method cannot be applied without anesthesia and dissection of hepatic vessels. Anesthesia reduces hepatic blood flow and, presumably, portal blood flow (Shackman, Graber and Melrose, 1953) and dissection of hepatic artery and portal vein may modify the portal fraction of hepatic blood flow in anesthetized dogs (Huet, P.M., unpublished data).

After shunt surgery, the hepatic blood flow generally decreases significantly, confirming the per-operative finding that the loss of portal flow is rarely completely compensated by an increase in the hepatic arterial flow (Warren, Restrepo, Repress and Muller, 1963; Reynolds, 1970). Pre-operative hepatic blood flow does not seem to carry a prognostic value in regards to the clinical course after shunt surgery (Reynolds, 1970; Smith, 1974); however, patients with a post-operative hepatic blood flow higher than 600 ml/min tended to have more satisfactory clinical results (Reynolds, 1970). It should be emphasized once more that no studies were performed, adding the pre-operative measurement of the portal fraction of hepatic blood flow to the other hemodynamic parameters.

Aside from these hemodynamic evaluations of the portal system, marked systemic abnormalities were reported in alcoholic cirrhosis of the liver in which a hyperkinetic circulation is a characteristic feature (Siegel, Goldwyn, Farrell, Gallin and Friedman, 1974). Diminished systemic vascular resistance, arterial oxygen unsaturation and evidence of multiple arteriovenous shunts have been demonstrated (Mellemgaard, Winkler, Tygstrup and Georg, 1963; Greenspan and Del Guercio, 1968; Martini, Arndt, Baltzer, Buchta, Hardewig, Marsch and Schmidt, 1970). Increased blood volume, with a normal red cell mass, has also been a well recognized feature of cirrhosis (Perera, 1946; Murray, Dawson and Sherlock, 1958). The pathogenesis of this plasma volume expansion has not been clearly established; however, a mechanical cause such as obstruction to the portal flow, whether intra or extra hepatic, appears to be at least partly responsible. Plasma volume measurements in cirrhosis have been found in correlation with those of wedged

hepatic venous pressure, and was significantly higher in patients with esophageal varices and a history of bleeding (Lieberman and Reynolds, 1967; Lefebvre, Joubert, Marleau, Joly, Bernier, Lavoie and Viallet, 1969). Increased hazard of bleeding from esophageal varices has also been found in cirrhotics whose plasma volume was increased with albumin infusions in the treatment of resistant ascites (Wilkinson and Sherlock, 1962).

UMBILICOPORTAL CATHETERIZATION

Gonzales-Carbalhaes (1959) first demonstrated that the collapsed umbilical vein of adult man remains connected to the left branch of the portal vein and can be dilated and cannulated. Many groups of investigators have further developed this technique (Bayly, 1964; Lavoie and Viallet, 1965; White, Slapak and MacLean, 1968; Chianidussi, 1970). Under epidural or, in some cases, general anesthesia, the round ligament of the liver can be identified extraperitoneally and catheterized into the portal vein under fluoroscopy. The use of umbilicoportal catheterization offers direct access to the portal system, allowing a more accurate investigation of the portal and hepatic circulation, in cases of portal hypertension.

Portal Circulation

Investigations of portal circulation can be considered under two headings: radiology and manometry.

Radiologic findings. Following portal catheterization, umbilico-portographies are taken, using a rapid film changer after injecting 20 to 50 ml of radiopaque material with a pressure injector (usually 60 lb per square inch). Angiographies are performed to visualize the extra and intra hepatic portal system.



Figure 4. Umbilico-splenography (3 sec) showing short gastric veins from splenic hilus with opacification of large esophageal varices (†); the coronary vein is dilated (▲) and intrahepatic portal branches are sparse and irregular.

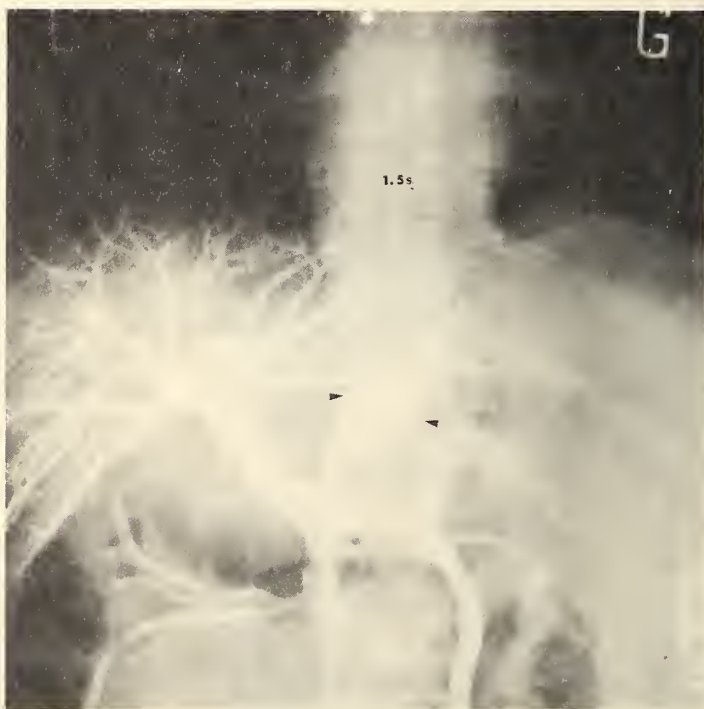


Figure 5. *a) Normal umbilico-portography (1.5 sec) in a patient with choledochus carcinoma.*

b) Umbilico-portography (1.5 sec) in a patient with alcoholic cirrhosis showing dilated coronary veins (▲) with gastric varices. The intrahepatic portal branches are sparse and irregular.



(b).



(a)

Figure 6. Umbilico-portographies in a patient with alcoholic cirrhosis, showing reverse circulation in the superior mesenteric vein and large mesenterico-splenic shunt (a and b) with opacification of the inferior vena cava (b).

Opacification of the extrahepatic portal system is obtained through retrograde opacification of the superior and inferior mesenteric veins, splenic vein and portal trunk. These porto-splanchnographies are occasionally obtained when the tip of the catheter is positioned close to the umbilicoportal junction. Therefore, Lavoie *et al.* (1967) have improved this technique by guiding the catheter with a metallic probe under fluoroscopy, thus realizing true selective umbilico-splenography (Figure 4), and umbilico-portography (Figure 5).

In alcoholic cirrhosis of the liver with portal hypertension, the coronary vein is generally opacified and dilated with or without inverted circulation through esophageal varices. Its involvement may be evaluated and graded (Viallet, Légaré and Lavoie, 1970b). Other collaterals contributing to the formation of gastroesophageal varices may be visualized only on umbilico-portographies, through short gastric veins arising from the splenic hilus (Figure 4), demonstrating the importance of this technique.

Other collateral derivations such as mesenterico-caval shunts (Figure 6) or spleno-ovaro-renal shunts (Figure 7) are less frequent, and not usually outlined by splenoportography. In some patients, reverse or stagnant flow in the portal vein, with a large spontaneous porto-systemic shunt, is demonstrated with delayed cavography (Figures 6 and 8). Spontaneous thrombosis of the portal vein has rarely been reported in umbilico-portographies (Kessler, Trice and Zimmon, 1973). In more than 150 cirrhotic patients, investigated in the last 8 years at Hôpital Saint-Luc, spontaneous thrombosis of the portal vein or its main tributaries was not found on umbilico-portographies.



Figure 7. Umbilico-splenography in a patient with alcoholic cirrhosis, showing large spleno-ovaro-renal shunt.



Figure 8. *Umbilico-portography in a patient with alcoholic cirrhosis, showing spontaneous splenorenal shunt with opacification of the inferior vena cava; lacunar defects are present on hepatogram.*

Opacification of the intrahepatic portal system may be divided into a venous phase (Figures 4 and 5) and a sinusoidal phase (Figure 9). In alcoholic cirrhosis, the intrahepatic portal vascularization is sparse and irregular, and ramifications, perpendicular to the main vessels (at 45° in normal liver), take on the aspect of a dead tree (Figures 4 and 5b). A somewhat mottled hepatogram has been described and probably results from hepatic scarring and regeneration (Figure 9). In some patients, it may be awkward to discriminate lacunar defects at the sinusoidal phase (Figure 8), from those caused by primary or secondary hepatic tumors on cirrhotic liver, when tumors are poorly irrigated by portal vessels. In such cases, only liver biopsy in a radiolucent area could give the diagnosis.

Pressure recordings. With the catheter tip in the main portal trunk, free portal venous pressure (FPVP) may be recorded; zero level is assumed to be 5 cm below the sternal angle, with the patient in a supine position. However, to eliminate the effects of variations resulting from systemic venous hypertension and/or intra-abdominal changes in pressure, a more reliable baseline has to be used (Iwatsuki and Reynolds, 1973). Joly *et al.* (1968), combining the umbilicoportal catheterization with hepatic vein catheterization, determined this baseline as FHVP and used the portohepatic gradient (FPVP less FHVP) as an index for portal hypertension. In the last 54 alcoholic cirrhotic patients investigated at Hôpital Saint-Luc, the porto-hepatic gradient varied between 7 and 26 mm Hg.



Figure 9. *Umbilico-portography (10 sec) in a patient with alcoholic cirrhosis, showing a mottled sinusoidal phase and stagnant flow in the splenic and inferior mesenteric veins.*

Prior to the use of umbilicoportal catheterization, values of wedged hepatic venous pressure (WHVP) were used as an estimate of portal pressure in cirrhosis. The reliability of data obtained by hepatic vein catheterization were subsequently demonstrated by identical or nearly identical values between simultaneous recordings of FPVP and WHVP in cirrhotic patients, as previously shown on Figure 1.

Portohepatic gradient can correlate with other, direct or indirect investigation tests or portal hypertension, such as the importance of coronary vein involvement on umbilico-portography (Viallet *et al.*, 1970b) and the size of esophageal varices at endoscopy (Aoun *et al.*, 1971). Figure 10 illustrates unpublished data in 38 alcoholic cirrhotic patients recently evaluated at Hôpital Saint-Luc. In all patients with ruptured varices, at emergency endoscopy, the portohepatic gradient was higher than 12 mm Hg, only one being below 14.5 mm Hg. Varices were graded 1 to 4 according to their diameter at endoscopy; in all patients with variceal bleeding, varices were graded 3 or 4 mm diameter or more. These findings suggest that patients with a high portohepatic gradient and large varices are to be considered as potential bleeders (Joly, Marleau, Légaré, Lavoie, Bernier and Viallet, 1971), even if both of these parameters cannot be used as diagnostic tools for a bleeding episode. However, a portohepatic gradient of 12 mm Hg or less (and small varices) should indicate that varices are not the source of upper gastrointestinal hemorrhage in alcoholic cirrhotic patients. These hemodynamic studies may be useful before considering surgical therapy in patients with varices, when the site of bleeding cannot be determined at emergency endoscopy.

A correlation was also found between the portohepatic gradient and liver function tests (Lefebvre *et al.*, 1969). In a series of 54 alcoholic cirrhotic patients, a significant negative correlation was found between the relative clearance of indocyanine green (K-ICG) and the portohepatic gradient (Figure 11). Millette *et al.* (1973a) also found a positive correlation between the extrahepatic uptake of radio colloidal gold (Au^{198} EHU) and the portohepatic gradient. However, even if a significant correlation has been found between K-ICG or Au^{198} EHU, and the portohepatic gradient, these parameters have a limited value in the assessment of portal hypertension in individual cases of cirrhosis.



Figure 10. Comparison of varices graded at endoscopy and porto-hepatic gradients in 38 alcoholic cirrhotics (O patients bleeding from ruptured varices; ● patients bleeding from non variceal site; ▲ patients without bleeding episode).

When compared to parameters of systemic circulation, a positive correlation has been found with the plasmatic volume in 54 alcoholic cirrhotics (Figure 12) (Viallet, unpublished data). Zimmon and Kessler (1974) reported that portal vein pressure varied directly with blood volume after increase or decrease of the intra-vascular volume, in patients with cirrhosis and portal hypertension. The cardiac output (or cardiac index), even though increased in cirrhosis (Siegel *et al.*, 1974), was not found to correlate with the portohepatic gradient in 33 alcoholic cirrhotics (mean cardiac output: 7.36 L/min; mean cardiac index: 4.22 L/min/m²) (Viallet, unpublished data).

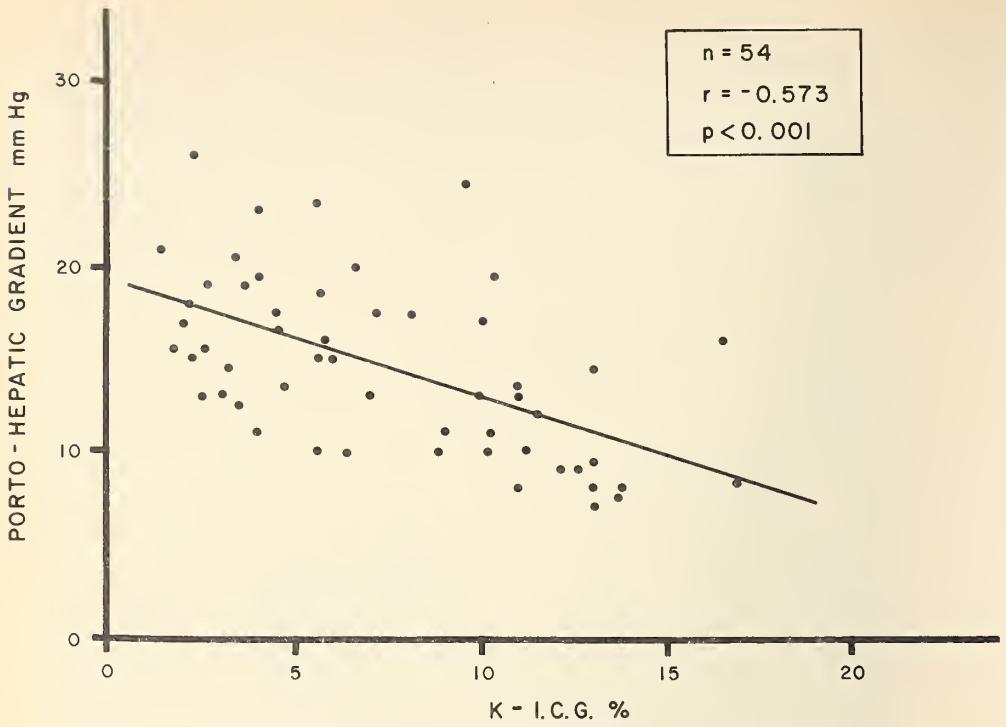


Figure 11. Comparison of the relative clearance of indocyanine green (K-ICG) and porto-hepatic gradient in 54 alcoholic cirrhotics.

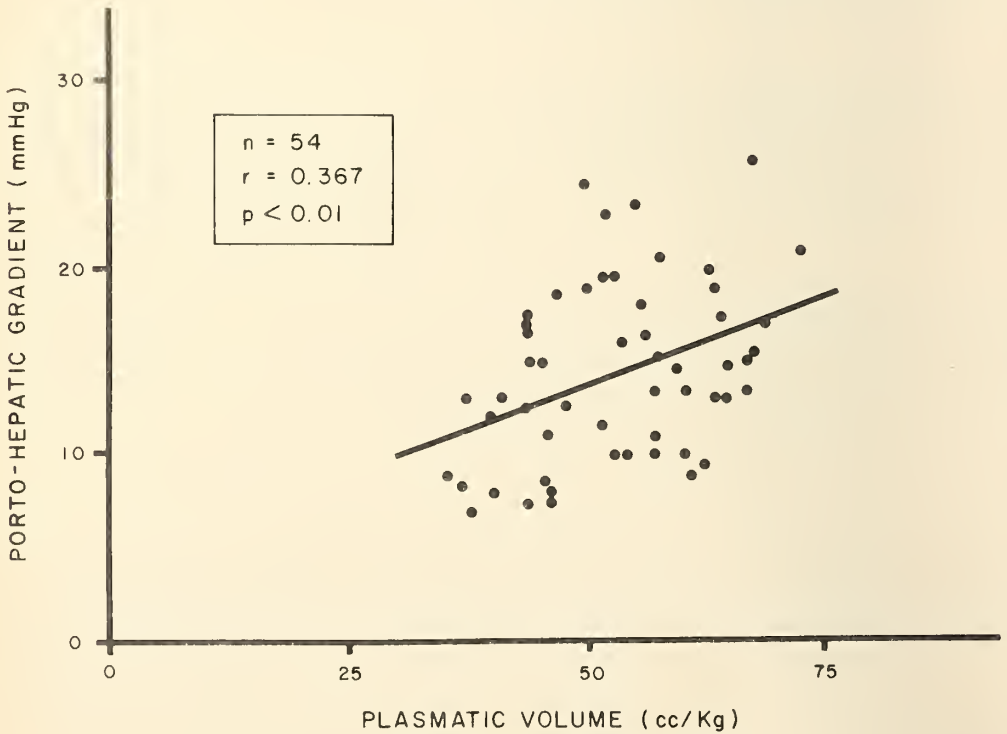


Figure 12. Comparison of the plasmatic volume and porto-hepatic gradient in 54 alcoholic cirrhotics.

Hepatic Circulation

Aside from the morphological aspect of hepatic vasculature described with umbilicoportography and measurement of total hepatic blood flow, the portal contribution to hepatic perfusion and function remains the major problem in the evaluation of hepatic circulation. In conscious patients, most indirect techniques have been devised to measure only the total hepatic blood flow, because of the dual supply to the liver and relative inaccessibility of the portal vein.

With the introduction of umbilicoportal catheterization, where samples from the portal vein are easily obtained, several methods have been described, estimating the portal blood flow or the portal fraction of total hepatic blood flow (Chiandussi, Greco, Sardi, Vaccarino, Ferraris and Curti, 1968; Stone, tenHove, Effros and Leevy, 1972; Reichle, Sovak, Soulen and Rosemond, 1972; Strandell, Erwald, Kulling, Lundbergh, Marions and Wiechel, 1972; Dencker, Göthlin, Olin and Tibblin, 1972; Huet, Lavoie and Viallet, 1973).

Chiandussi *et al.* (1968) first applied the Stewart Hamilton principle to evaluate simultaneously portal and hepatic blood flows in cirrhotic patients. Following intra-splenic injection of I^{131} labelled serum albumin, indicator dilution curves were recorded simultaneously from the left branch of the portal vein and from one hepatic vein. The ratio of portal blood flow over hepatic blood flow could be calculated by comparing curves obtained from portal and hepatic veins. In 8 cirrhotics, the mean portal fraction was 60.5%, although it was 69.6% in 3 normal patients. Intrasplenic injection was subsequently obviated using a double lumen umbilicoportal catheter: the distal opening being the injection site and the proximal opening the portal sampling site (Curti and Chiandussi, 1971). However, these approaches have not been controlled in an experimental model.

Stone *et al.* (1972) described a dilution method, advocating the Fick's principle which uses radioactive Xenon, a substance not excreted or metabolized by the liver, but almost completely removed during one passage through the lungs (more than 95%). In a preliminary study in alcoholic cirrhotic patients, the portal fraction of hepatic blood flow was evaluated by comparing simultaneous concentrations of radioactive Xenon in portal and hepatic veins, after injection of the indicator into the stomach. In 13 alcoholic patients with portal hypertension, the portal fraction varied from 0 to 88% of the hepatic blood flow. Reichle *et al.* (1972) have described a method measuring only portal blood flow in man. Portal blood flow was estimated, using the velocity of lipiodol droplets recorded on high speed cinefluoroscopy and a cross section area of the portal vein assessed by biplan portography. In 3 alcoholic cirrhotics, the mean portal blood flow was 614 ml/min. However, no information could be obtained simultaneously, concerning the total hepatic blood flow.

Recently, Huet *et al.* (1973) developed an indicator dilution method, using portal and hepatic indicator dilution curves after injection of Cr^{51} labelled red blood cells into the cranial mesenteric artery (Figure 13). Portal blood flow, total hepatic blood flow and the portal fraction of total hepatic blood flow were estimated in normal dogs. This method was validated by comparison with a direct method of measurement using electromagnetic flowmeters. This technique was applied in 13 compensated alcoholic cirrhotic patients with severe portal hypertension, undergoing combined umbilicoportal, hepatic vein and superior mesenteric artery catheterization, measuring portal fraction of hepatic blood flow. Samples were obtained simultaneously from the portal bifurcation, one right hepatic vein, and when possible, from a left hepatic vein after injection of Cr^{51} labelled red blood cells into the superior mesenteric artery.

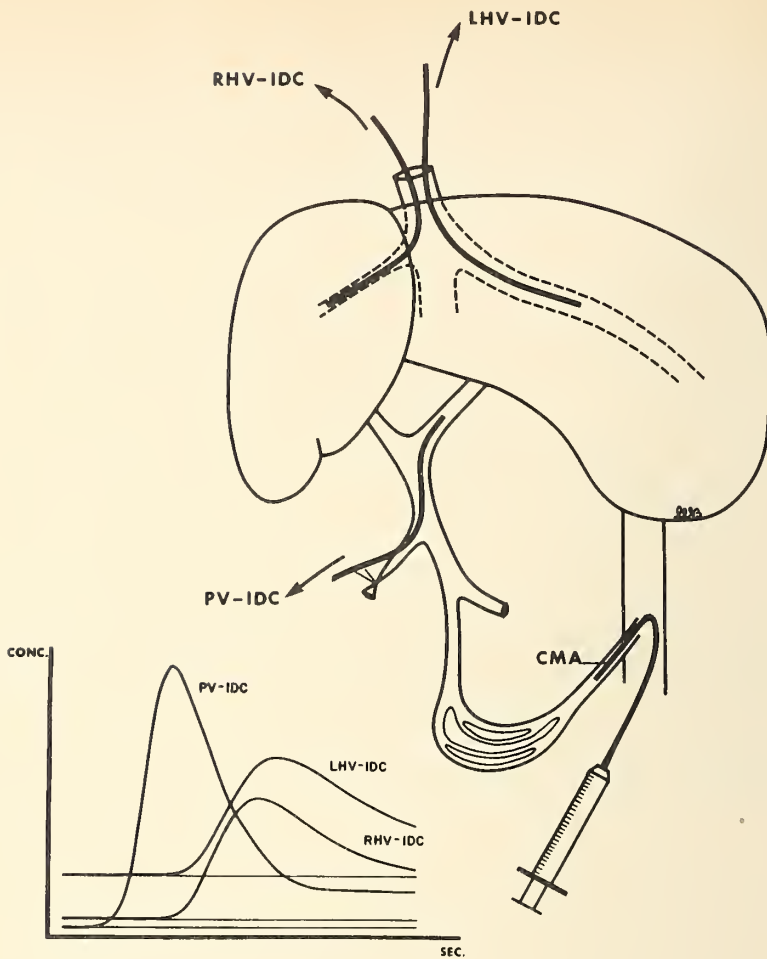


Figure 13. Schematic diagram showing the injecting site into the cranial mesenteric artery (CMA) and indicator dilution curves (IDC) obtained simultaneously from portal vein (PV), a right hepatic vein (RHV) and a left hepatic vein (LHV) in dog.

In cirrhotic patients, flows are overestimated because of loss of an unknown part of the indicator, through spontaneous portosystemic collaterals. However, no extrahepatic shunts were present in the studied patients after the portal bifurcation, as shown on portography. The same amount of indicator should be analysed at the bifurcation of the portal and hepatic vein. Therefore, even though absolute flow values cannot be measured, the ratio between the area of hepatic and portal indicator dilution curves can be used accurately for the measurement of the portal fraction of hepatic blood flow. In 10 patients, where calculable curves were obtained, the portal fraction (PF) varied from 30.1 to 100.3% (mean 71.1%) (Figures 14 and 15). In 4 of these patients, the mean difference between paired curves obtained simultaneously from two hepatic veins was 15.1% (7-30%), suggesting adequate mixing of the indicator in the portal vein as well as within the intrahepatic correlation, as demonstrated in normal dogs (Huet *et al.*, 1973). In 3 other patients, only delayed activity from recirculation was detected from portal and hepatic vein samples (Figure 16). A reverse and/or stagnant flow in the portal veins was found on portography and arteriography (Figures 7 and 8). In the same 3 cases, PF was 0%.

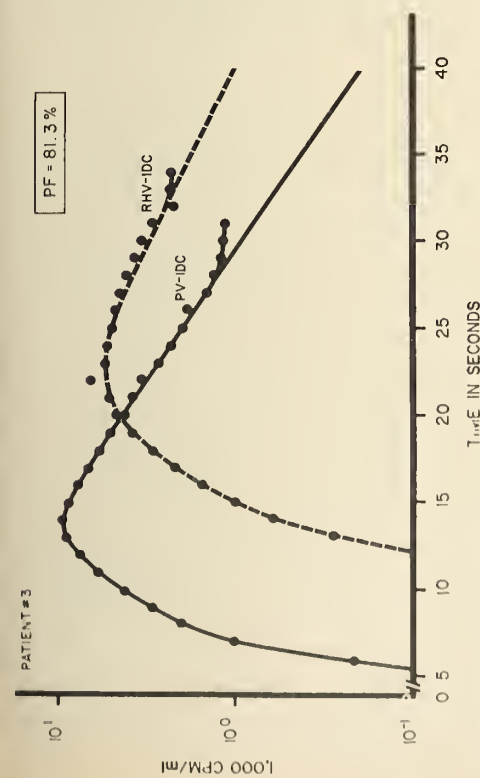


Figure 14. Simultaneous indicator dilution curves (IDC) obtained from the portal vein (PV) and a right hepatic vein (RHV) after injection of Cr^{51} red blood cells into the superior mesenteric artery in a patient with alcoholic cirrhosis (PF: portal fraction of hepatic blood flow).

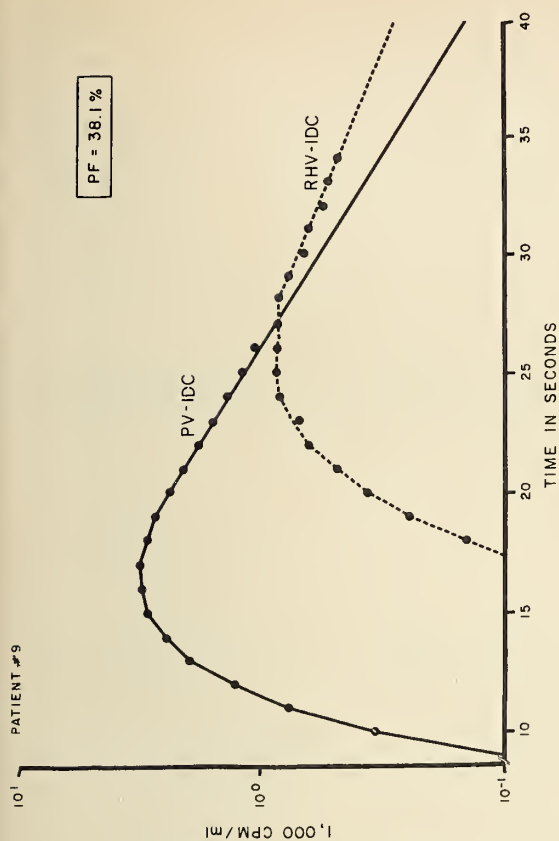


Figure 15. Simultaneous indicator dilution curves (IDC) obtained from the portal vein (PV) and a right hepatic vein (RHV) after injection of Cr^{51} red blood cells into the superior mesenteric artery in a patient with alcoholic cirrhosis (PF: portal fraction of hepatic blood flow).

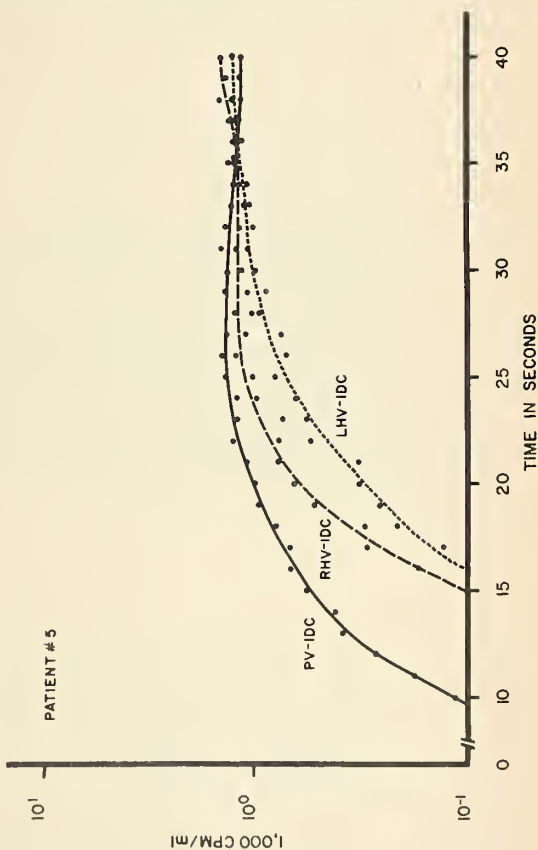


Figure 16. Activity recorded in samples obtained from the portal vein (PV-IDC), a right hepatic vein (RHV-IDC) and a left hepatic vein (LHV-IDC) after injection of Cr^{51} red blood cells into the superior mesenteric artery in an alcoholic cirrhotic with reverse circulation in the portal vein.

In the 13 patients, no correlation existed between the portal fraction of hepatic blood flow and the K-ICG or the portohepatic gradient. No correlation has been found with estimated hepatic blood flow, using continuous perfusion of indocyanine green. If confirmed, these data will indicate that this method can be used for the estimation of the portal fraction of hepatic blood flow in cirrhotic patients before portacaval shunts. Thus, one may determine whether any critical level of portal fraction exists above which portacaval shunting would give poor clinical results.

Special Studies

Umbilicoportal catheterization allows an accurate evaluation of portal hypertension in alcoholic cirrhosis of the liver, but it also allows a biochemical and physiological approach to this entity.

Joly *et al.*, (1967), combining umbilicoportal and hepatic vein catheterization, found that there was no difference in the catecholamine levels in portal, hepatic and peripheral venous blood in cirrhotic patients, as well as in normal subjects. Dufault *et al.*, (1970) found a significant fall in hepatic venous free fatty acid concentrations in cirrhotics when compared to normal subjects, but no significant differences were found between the coagulation factors in portal and hepatic venous blood in cirrhotics. Using combined catheterization, it was shown that the active stereoisomer of folinic acid (5-formyltetrahydrofolate) undergoes metabolism mainly during absorption through the intestinal wall and not within the liver, in non cirrhotic as well as in cirrhotic patients (Whitehead, Viallet and Cooper, 1972). The insulin response to glucose, secretin and glucagon, were studied in the portal and peripheral veins in normal and cirrhotic patients (Dupré, Rojas, White, Unger and Beck, 1966; Blackard and Nelson, 1970; Erwald, Hed, Nygren, Røjdmark, Sundblad and Wiechel, 1971); a rough correlation between the portal and peripheral venous insulin response was reported with a 40-50% insulin extraction by the liver.

Decrease in portal venous pressure (20-45%) has been reported after systemic intravenous administration of vasopressin (10-20 units infused in 10-30 minutes) (Silva, Moffat and Walt, 1969; Viallet, Bernier, Marleau and Lavoie, 1970c) used successfully in the control of variceal bleeding in patients with portal hypertension (Schwartz, Bales, Emerson and Mahoney, 1959; Shaldon and Sherlock, 1960). This technique has become less popular because of the potential systemic adverse effects (mainly on the cardiac output and coronary blood flow). Selective inferior mesenteric artery perfusion of vasopressin (.2 to .4 units/min) was proposed to prevent the systemic effects and to obtain maximal mesenteric arteriolar vasoconstriction (Nusbaum, Baum, Sakiyalak and Blakemore, 1967). However, in 8 cirrhotic patients with portal hypertension, Millette *et al.* (1973b) reported that the mean decrease of portal pressure, even though significant, was only 9.6% during selective inferior mesenteric artery perfusion of vasopressin (.2 units/min). These data suggest that if selective vasopressin perfusion is efficacious in controlling bleeding varices, the therapeutic effect cannot be totally explained by lowering portal pressure in cirrhotic patients with portal hypertension. Moreover, the significant decrease of portal PO₂ (Millette *et al.*, 1973b), systemic effects (Millette *et al.*, 1973b) and vascular thrombosis (Renert, Button and Fuld, 1972) reported with the use of this technique have to be considered in cirrhotic patients with ruptured varices.

Porto-systemic shunts via the reopened umbilical vein and saphenous (or external jugular) vein were performed to obtain a temporary or permanent decompression of the

portal system (White *et al.*, 1968; Piccone, Bonanno and Leveen, 1968; Kessler *et al.*, 1973). Successful control of variceal bleeding has been reported in patients whose shunt remained patent (White *et al.*, 1968; Kessler *et al.*, 1973). This technique was proposed to filter parasites from the portal blood in schistosomiasis (Kessler, Amadeo, Tice and Zimmon, 1970).

Zimmon and Kessler (1971) used the extracorporeal umbilical saphenous shunt to study the response of the "hepatic sinusoidal pressure" (as evaluated by the WHVP or FPVP) to portal venous flow diversion in cirrhotic patients before portacaval shunts. In some cirrhotic patients (type A), as well as in normal subjects, the pressure is maintained while in other cirrhotic patients (type B), marked falls are recorded during extracorporeal shunting. It was assumed that pressure variations are secondary to hepatic arterial response to portal flow diversion. A preliminary report suggests that patients showing reduced "hepatic artery response" (type B) do poorly after portacaval shunts, while type A patients tolerate the procedure well (Kessler, Tice and Zimmon, 1972).

Complications

Three rare but serious complications have been reported following umbilicoportal cannulation: infection (Johns, Kitchen and Leslie, 1972), bleeding (Silva, 1970) and portal thrombosis (Kessler *et al.*, 1973). In a series of over 400 procedures (Lavoie, personal communication), no wound infection was observed, but in a few cases, the body temperature rose in the following days; however, blood cultures were sterile and recovery was uneventful in all patients without antibiotic treatment. Back bleeding from portal vein into the peritoneal space was never encountered. In only one patient, a partial thrombosis in the right branch of the portal, not found on initial portography, was reported at autopsy, six months after cannulation.

CONCLUSION

Portal hypertension following changes in hepatic circulation remains the major clinical problem in the management of patients with alcoholic cirrhosis of the liver. Medical and surgical treatments of variceal hemorrhage is still associated with a high mortality rate. If "potential bleeders" can be selected by measuring a portohepatic gradient higher than 12 mm Hg (and the presence of large varices at endoscopy), the diagnosis of a bleeding episode from ruptured varices can only be done during emergency endoscopy. However, a portohepatic gradient lower than 12 mm Hg should indicate that varices are not the source of upper gastrointestinal bleeding.

Portacaval shunts have proven to be efficacious in preventing recurrent bleeding but the loss of portal blood flowing into the liver may aggravate the hepatic failure and is associated with a high incidence of encephalopathy. So far, most of the splanchnic hemodynamic measurements do not correlate with the post-operative course. However, it should be determined whether any critical level of the portal fraction of hepatic blood flow exists above which portacaval shunting would give poor clinical results; furthermore, the functional hepatic blood flow (or the effective flow to the hepatocytes) has not been evaluated pre-operatively. Eventually, portacaval shunting could be reserved for patients whose portal fraction (and/or functional blood flow) is reduced. In patients with a high portal fraction (and/or high functional blood flow) distal splenorenal shunts (Warren,

Fomon and Zeppa, 1969; Warren, Salam, Faraldo, Hutson and Smith, 1972) or spleno-caval (Texeira, Uyu, Conn and Bergan, 1968) may provide less diversion of portal flow while relieving portal hypertension. Arterialization of the portal vein, associated with portacaval shunting, may be an alternative to preserve hepatic blood flow; preliminary reports are encouraging (Adamson, Kinkhabwala, Moskowitz, Himmelfarb, Minkowitz and Lerner, 1962; Maillard, Rueff, Prandi and Sicot, 1974).

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The Course of Alcoholic Hepatitis

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INTRODUCTION

Ethanol is the most common drug of abuse in America. Alcoholic liver disease is by far the most common type of drug-induced liver injury. A conservative estimate is that over 3 per cent of the population in the United States are significantly affected by their excessive drinking. In Atlanta, Georgia, with a population of half a million, we have about 39,000 arrests for public drunkenness per year. The annual consumption of distilled spirits in the U.S. increased by 17-1/2 million gallons in 1972 and now exceeds 400 million gallons.

Alcoholic hepatitis is a chronic (it lasts months to years) disease of the liver in which the inflammatory response is secondary to parenchymal necrosis. Liver injury is due to ethanol and is not due to the other congeners also known to be present in alcoholic beverages.

The proponents of nutritional injury as a cause of alcoholic liver disease ignored pertinent observations. For example, the high incidence of alcoholic liver disease in France and Chile is related to the high per capita consumption of alcohol (wine) in these two countries and not to the vastly different nutritional status of the middle class French versus the impoverished Chilean drinking population. The decrease of alcoholic liver disease in France during World War II was related to the decrease of alcohol consumption despite the worsened nutrition of the French people; conversely, the rise of alcoholic liver disease after the war was associated with increased alcohol consumption together with improved nutrition in France. In the United States, the common denominator between alcoholic liver disease among executives and the charity patients is alcohol consumption, not their diet.

Cirrhosis is one of the four leading causes of death in the United States and alcoholic hepatitis is the most common cause of cirrhosis. Because alcoholic hepatitis is a reversible disease, it is evident that at some stage in the pathogenesis of alcoholic hepatitis-cirrhosis, this major cause of death can be prevented.

THE INCIDENCE OF ALCOHOLIC HEPATITIS

Until prospective and properly controlled epidemiologic studies will become available, the true incidence of alcoholic hepatitis in the general population and the relationship between the amount and duration of drinking of alcoholic beverages and alcoholic liver disease will not be known. Current information is available on patients who presented themselves for therapy. These patients fall in three major categories: 1) those whose presenting symptoms related to alcoholism, 2) those whose symptoms related to liver disease, and 3) those whose symptoms related to problems other than that of liver disease or alcoholism. Estimates based on such experience are likely to overestimate the frequency of alcoholic hepatitis. Another problem is that the mechanism of the parenchymal necrosis is not known. Such essential information will be delayed until an experimental model among primates becomes available. Encouraging new observations make this model a likely possibility.

The estimation of the incidence of alcoholic hepatitis among "heavy drinkers" is difficult. On the one hand, we observed the development of alcoholic hepatitis after only a few months of excessive drinking in young people; on the other hand, we have seen a large number of alcoholic derelicts on very inadequate diets who did not develop liver injury in spite of decades of alcohol abuse. Nevertheless, 10 per cent is a conservative estimate of the incidence of cirrhosis among excessive drinkers. Our observations suggest that cirrhosis develops in about a third of the patients who have alcoholic hepatitis: it is reasonable to assume then that about a third of excessive drinkers have alcoholic hepatitis (about 20 million people in the U.S.).

PATHOGENESIS OF ALCOHOLIC HEPATITIS

Despite the occasional patient in whom this lesion developed after drinking for less than a year, alcoholic hepatitis developed in most patients only after 5 or more years of drinking. It is not likely that alcoholic hepatitis would develop in those who drink less than half a pint of whiskey or one liter of wine (about 80 grams of alcohol) per day. In such persons, ethanol provides less than 20 per cent of their daily calorie intake. When the daily consumption is equivalent to half a pint to a pint of whiskey and the duration of drinking is prolonged, then the risk of alcoholic hepatitis is increased. Probability that this person will develop alcoholic liver disease is much greater when the alcohol consumption exceeds the metabolic capacity of the liver of an average non-drinking person, which is approximately 160 grams per day, and when his drinking has exceeded this capacity for a decade or more. The evidence is quite persuasive that the greater the amount of alcoholic beverages consumed and the longer the duration of drinking, the higher the frequency of alcoholic liver disease.

It is important to recognize the fact that drunkenness is not a prerequisite for alcoholic liver disease, although alcohol consumption is. It is well-known that alcoholic hepatitis can develop in economically successful, productive and active individuals who

are quite successful in their chosen profession or business. These persons can perform effectively in their area of responsibility without showing any overt signs of intoxication despite the fact that they consume large amounts of alcoholic beverages. It is interesting in this respect that in the United Kingdom alcoholic cirrhosis is a disease of the well-to-do, while in the United States it is more commonly reported from institutions dealing with charity patients. At least 5 per cent of those patients with alcoholic liver disease who were studied in London, England, were physicians. It is likely that as the rate of ethanol metabolism increases in a drinking person, his ability to imbibe larger amounts of his favorite brew also increases without a corresponding rise of his blood ethanol concentration over the legal limit of intoxication (about 80 mg/100 ml which is the limit of the conventional breath analyzer test used by law enforcement agencies).

Whether the pathogenesis of alcoholic hepatitis is related purely to the duration and the amount of ethanol consumed is not clear. Indeed, the evidence indicates that the "dose response curve" in human alcoholics may be rather crude. Because drinks contain, in addition to ethanol, other types of alcohols and organic compounds, it was suggested that these congeners may well contribute significantly to the development of what is called alcoholic liver disease. On the basis of epidemiologic observations, the worldwide distribution of alcoholic liver disease seems to be related to consumption of ethanol and not to any particular type of brew. There is a more readily discernible association of alcoholic liver disease and the consumption of wine and beer than that of bourbon or scotch. The latter are rich in these organic contaminants — or flavours. It is not likely that the additional organic contaminants in wine, beer and different types of whiskey are similar. Furthermore, the drinking of essentially pure dilute ethyl alcohol (vodka) is also associated with alcoholic liver disease. These considerations make the theory unlikely that a particular trace contaminant rather than ethanol itself is responsible for alcoholic liver disease.

It is possible, however, that some acquired or genetic characteristics may increase susceptibility to ethanol-induced parenchymal necrosis and alcoholic liver disease. One of the important epidemiologic observations which indicate a constitutional disposition to alcoholic liver disease is the sex-linked susceptibility to alcoholic liver injury. This characteristic was first suggested in the 1940s, and subsequent data supported the observations that alcoholic liver injury is more common among women. Our observations documented that a much higher proportion of black women developed alcoholic hepatitis than would be expected by chance alone on the basis of the sex-race distribution of patients in Grady Memorial Hospital in Atlanta, Georgia. It is important to note in this respect that the mortality rate of alcoholic hepatitis among women is higher than that among men. At the time of death from alcoholic liver disease the average age of women is significantly lower than that of men. The proportion of women with alcoholic cirrhosis under the age of 40 is greater than that of men. We have observed that the mean age of women was significantly younger at the time of the biopsy diagnosis of alcoholic hepatitis than that of men. And, finally, the rate increase of mortality from cirrhosis in the United States for black women is higher than that of black men or of whites.

THE COMPOSITION OF THE HEPATIC CONNECTIVE TISSUE IN ALCOHOLIC LIVER DISEASE

Connective tissue composition was studied in patients with alcoholic liver disease and in normal controls. Livers were collected at autopsy, were sectioned and fixed in acetone promptly to prevent autolysis. Chemical analysis was then performed on acetone and

ether dried defatted liver (DDL). This study includes 10 normal livers, 5 livers with severe alcoholic steatosis (fatty livers), 7 livers with alcoholic hepatitis and fibrosis but no cirrhosis, and 10 livers with cirrhosis but no overt alcoholic hepatitis.

Collagen. — The total collagen content (hydroxyproline) and the molar sodium chloride soluble collagen concentrations were determined and are described in Table I. The total insoluble collagen concentration in the fatty liver was similar to that of normal. However, both were increased in alcoholic hepatitis and in alcoholic cirrhosis ($p < 0.001$). The total collagen concentration in alcoholic hepatitis and cirrhosis were similar; however, the soluble collagen concentration in alcoholic hepatitis was almost twice that of cirrhosis. It is noteworthy, nevertheless, that there was a significant increase of soluble collagen in those cirrhotic livers which did not show clear-cut evidence of inflammation and parenchymal necrosis, indicating a considerable increase of recently synthesized collagen in the connective tissue of the alcoholic (Laennec's) cirrhotic livers.

TABLE I
COLLAGEN CONTENT OF NORMAL LIVER
AND IN ALCOHOLIC LIVER DISEASE

	N	Total Collagen*	N NaCl Extractable Collagen
Normal	10	6.9† ± 0.37	0.13 ± 0.06
Fatty Liver	5	7.2 ± 1.5	0.4 ± 0.12
Alcoholic Hepatitis	7	28.1 ± 3.1	2.01 ± 0.39
Cirrhosis§	10	27.4 ± 2.8	1.09 ± 0.21

N = number of livers per diagnostic category
 * mg hydroxyproline per gm dried defatted liver
 † mean ± S.E. of livers per diagnostic category
 § all visible fibrous scar tissue was excluded

Note: The data presented in this Table and in Table II are mean ± S.E. of each diagnostic group. The average estimate of replicate analyses per liver was used to compute the mean per group. The number of livers in each group and not the number of samples analyzed per liver was used as "N" and "D.F." in statistical computations.

The other component of the hepatic connective tissue which was studied was the glycosaminoglycuronans (GAG). The native state of these polysaccharides is a protein polysaccharide complex of high molecular weight (proteoglycans).

Soluble GAG. — Under gentle conditions with a tris pH 8.2 buffer, the proteoglycans were extracted and studied separately. The non-extractable GAG were isolated after proteolytic digestion of the remaining liver. For each diagnostic category, the total soluble and insoluble GAG are described in Table II.

The proteoglycans were separated according to their isoelectric pH (pI). The proteoglycans had a pI ≤ 3.1 and the major fraction had pI ≤ 2. In livers with alcoholic hepatitis there was not only a marked increase of the extractable proteoglycans, but this increase was due to the fraction with pI = > 2 < 2.75. Examination of the GAG component of the proteoglycans showed that both normal livers and alcoholic hepatitis livers contained hyaluronidase resistant heparan sulfate and dermatan sulfate in similar concentrations.

The amino acid composition of the pooled extractable proteoglycans with $pI \leq 3.1$ from normal livers was similar to that from alcoholic hepatitis; the amino acid compositions of proteoglycans which were obtained from livers were not different from proteoglycans obtained from cartilage.

Insoluble GAG. — The concentration of insoluble GAG in alcoholic hepatitis and cirrhosis was greater than that in fatty liver and normal liver ($p < 0.05$; Table II). The predominant GAG in all livers were chondroitin-4 and chondroitin-6-sulfate. There was no evidence that alcoholic hepatitis or cirrhosis contained larger amounts of hyaluronidase resistant dermatan sulfate or heparan sulfate as compared to normal liver. There was, however, a significant increase of hyaluronic acid in alcoholic hepatitis and also that of an unidentified hyaluronidase resistant GAG. The electrophoretic mobility of the unidentified, hyaluronidase resistant GAG was similar to hyaluronic acid. Of great interest is the observation that in the plasma of some patients with alcoholic hepatitis under the same conditions a GAG was detected which had similar electrophoretic mobility and was resistant to hyaluronidase hydrolysis.

DIAGNOSIS OF ALCOHOLIC HEPATITIS

Although the diagnosis of alcoholic hepatitis can be strongly suspected on clinical grounds, it can be established with certainty only on morphologic examination of the liver. If the liver biopsy is contraindicated on clinical grounds because of prolonged prothrombin time, the combination of the following findings on the data base are indicative of alcoholic hepatitis: 1) prolonged and recently excessive drinking of alcoholic beverages, 2) anorexia, 3) nausea with or without vomiting, 4) the demonstration of an enlarged liver which is smooth and not nodular; it may or may not be tender, 5) serum bilirubin over 5 mg/100 ml, 6) SGOT of less than 500 U, 7) serum alkaline phosphatase (SAP) is usually elevated but not over 3 times its upper limit of normal for the laboratory, 8) serology is negative for hepatitis B antigen. The following findings give additional strong support for the diagnosis of alcoholic hepatitis: 1. *Clinical* — abdominal pain, unexplained fever, palpable spleen and ascites. 2. *Laboratory* — anemia, leukocytosis and a very low BUN in the presence of normal serum creatinine clearance (if ascites develops and the creatinine clearance decreases, the BUN may appear to be normal or high).

Despite the typical and characteristic clinical manifestations of alcoholic liver disease, the diagnosis of alcoholic hepatitis was often delayed. Even after most of the signs and symptoms of alcoholic hepatitis are fully developed, the diagnosis was made within 6 months only in two-thirds of the patients and it was delayed for more than 4 years in over 10 per cent of the patients. It is important to stress, however, that the milder the clinical manifestations of the disease, the longer the delay between its appearance and its proper recognition and diagnosis; conversely, the more severe the clinical manifestations of alcoholic hepatitis, the shorter the elapsed time between its appearance and diagnosis. It was also pointed out that the diagnosis is made more rapidly in private (paying) patients. The duration of alcoholic hepatitis was significantly longer in those patients who had cirrhosis on their liver biopsy than in those in whom cirrhosis had not yet developed. The most common causes of "error" in not recording the diagnosis of alcoholic hepatitis is the physician's erroneous assumption that liver disease in the alcoholic is cirrhosis or that a transient episode of liver disease in a drinking person is due to fatty liver. The error in both instances was because a liver biopsy was not obtained (or that it was improperly interpreted). The diagnosis of alcoholic hepatitis many times is not recorded on hospital charts or on the medical record because of non-medical pressures and considerations.

Not making the diagnosis of alcoholic hepatitis may lead to the important, and at times fatal, error of making an erroneous diagnosis. The patient who had jaundice, leukocytosis, fever, severe (often right upper) abdominal pain, nausea and vomiting, and right upper quadrant tenderness, whose SAP is elevated and whose SGOT is only mildly increased (all these are characteristic findings of alcoholic hepatitis) can be mistaken for biliary tract disease and the patient can be operated on in error. The mortality rate of surgery is excessively high indeed in patients who have acute inflammation and necrosis in the liver, whether it is alcoholic hepatitis or viral hepatitis.

The clinical picture of alcoholic hepatitis. — On one extreme the liver biopsy diagnosis of alcoholic hepatitis has been made in patients who were asymptomatic and whose liver function tests were normal and the only reason for the biopsy is the palpable liver and history of drinking. On the other extreme, alcoholic hepatitis can run a course indistinguishable from that of fulminant hepatic failure due to drugs (halothane) or viral hepatitis.

TABLE II
EXTRACTABLE (PROTEOGLYCANS) AND NON-EXTRACTABLE GAG
HEXURONIC ACID — μg per 10 gm DDL

	Alcoholic			
	Normal (10)	Fatty (5)	Hepatitis (7)	Cirrhosis (10)
Soluble**	243* \pm 55	178 \pm 68	744§ \pm 151	310 \pm 89
Insoluble	325 \pm 61	273 \pm 83	492† \pm 47	713† \pm 160
Sum of means	568	441	1236	1023

*mean \pm S.E. of livers per diagnostic category
**soluble in pH 8.2 tris buffer
Probability that the observed difference from normal liver was due to chance:
†p < 0.05
§p < 0.01
The observed difference of extractable GAG between alcoholic hepatitis and cirrhosis was due to change:
p < 0.05, and that of non-extractable GAG: p = 0.19
(N) = number of livers per diagnostic category
See Note from Table I.

Indeed, one cannot ignore the impressive similarity between the spectrum of the clinical manifestations of the two most common types of liver diseases in the United States: that of alcoholic and of viral hepatitis. In both types of disease, the clinical course can range from the anicteric and asymptomatic on one hand to fulminant on the other. Both can run a mild but prolonged course which can be reversible, and both can run a chronic, aggressive course which may lead to cirrhosis or death.

Although some patients may have moderately severe morphologic lesions on the liver biopsy with very little or insignificant clinical findings, in general the severity of the clinical and morphologic manifestations of alcoholic hepatitis are reasonably well correlated.

The most common symptoms of alcoholic hepatitis are: anorexia, nausea, vomiting, abdominal pain and weight loss. The most common physical findings are: hepatomegaly, jaundice, fever, ascites and an enlarged spleen. The frequency of the symptoms and signs of alcoholic hepatitis are listed in Tables III and IV. Evidence of malnutrition is common among patients with alcoholic hepatitis in community hospitals but not as frequent among the more affluent.

TABLE III

THE AVERAGE INCIDENCE OF THE MOST COMMON
SYMPTOMS OF ALCOHOLIC HEPATITIS
IN 6 REPORTED SERIES OF PATIENTS

	%
Anorexia	70
Weight loss	55
Abdominal pain	50
Nausea and vomiting	40

TABLE IV

THE AVERAGE INCIDENCE OF THE MOST COMMON
SIGNS OF ALCOHOLIC HEPATITIS
IN 8 REPORTED SERIES OF PATIENTS

	%
Hepatomegaly	90
Jaundice	50
Fever	45
Ascites	40
Splenomegaly	30
Encephalopathy	15

The laboratory findings in alcoholic hepatitis. — The most common abnormalities are listed in Table V.

a. Abnormal wbc: Leukocytosis is more common among patients with severe than with mild disease. About half the patients who had severe and about a third of the patients with moderately severe alcoholic hepatitis on biopsy had elevated wbc. Leukopenia can be seen either in those patients with alcoholic hepatitis who have hypersplenism or who have a transient leukopenia because of ethanol induced depression of the marrow. It is noteworthy that the PMN leukocyte response is always suppressed in liver secondary to ethanol induced necrosis in patients whose liver biopsies are obtained right after an alcoholic binge.

b. Anemia: This is more marked in those who have poor economic and nutritional background and is seen more often in patients in city-county hospitals than on a private service.

c. Platelets: These are usually in the normal range; although 10 to 15% of the patients may have platelets less than 100,000 cu mm.

d. Liver function tests: The four most commonly used liver function tests in the identification of alcoholic liver disease are SGOT, SAP, serum bilirubin and serum proteins. The SGOT is normal in about a fifth of the patients; it is over 500 U in less than 1% of patients and is over 300 U in less than 5% of the patients. In general, SGOT is higher than the SGPT in alcoholic liver disease. The SAP is commonly elevated but it is rarely about 3 times upper limits of normal. Such elevation is seen in 60 to 80% of the patients with proven alcoholic hepatitis. Serum bilirubin remains within normal range in 10 to 40% of patients with alcoholic hepatitis. When the serum bilirubin is over 5 mg/100 ml, it usually indicates severe disease and often is associated with a prolonged prothrombin time. Serum proteins must be examined by electrophoresis because the results reported on the multiphasic analysis (SMA-12, 14, 18, etc.) are commonly erroneous. The serum albumin is often depressed and the serum gamma globulin is elevated in 50 to 75% of the patients. Among the immunoglobulins, it is the IgA which shows the most marked elevation in alcoholic hepatitis. This elevation of the IgA is characteristic of alcoholic liver disease. The increased gamma globulin is frequently seen in patients who have not developed cirrhosis.

e. The prothrombin time: This does not give diagnostic information about alcoholic hepatitis, but its prognostic value is not paralleled by any other single laboratory test or clinical finding.

TABLE V

THE AVERAGE INCIDENCE OF THE MOST COMMON
LABORATORY ABNORMALITIES OF ALCOHOLIC HEPATITIS
IN 5 REPORTED SERIES OF PATIENTS

	%
Anemia	70
Leukocytosis	45
SGOT — normal	20
< 500 U	99
< 300 U	95
Alkaline phosphatase	
(< X 3 elevation)	70
Bilirubin	75

PROGNOSIS OF ACUTE ALCOHOLIC HEPATITIS

The excessively prolonged prothrombin time which does not respond to specific therapy within 2 to 3 days indicates a high mortality rate. A prothrombin time which remains within 3 to 4 seconds of normal indicates a low probability of death during the acute illness. When the prothrombin time was close enough to normal to perform a liver biopsy, there were 47 deaths among 626 patients, a mortality rate of 7.1 per cent. In contrast, 42

of the 100 patients in whom liver biopsy could not be performed safely because of prolonged prothrombin time died. In a group of 169 patients in whom the prothrombin time was normal initially and a liver biopsy proved the diagnosis, about 15% deteriorated on "good" hospital management and abstinence. Their prothrombin time became progressively longer. Although the overall mortality rate of the 169 patients was 4%, the mortality rate in those in whom the prothrombin time became excessively prolonged was 20% as compared to the mortality rate of 1% of those in whom the prothrombin time remained normal.

1. *Jaundice.* — An elevated serum bilirubin did not have an independent prognostic value in those patients in whom the diagnosis of alcoholic hepatitis was made by liver biopsy. One of the possible reasons why it is commonly assumed that jaundice has a prognostic value in alcoholic hepatitis is the fact that many studies were made on patients who were selected just because they were jaundiced or were selected from autopsy files (which has a rather poor prognosis even in non-icteric cases).

2. *Liver biopsy.* — Among patients with alcoholic hepatitis, the severity of the morphologic liver injury roughly relates to prognosis; however, the presence or absence of alcoholic hyalin has no prognostic value.

3. *Long term prognosis.* — Those patients who survived the acute clinical illness and made a reasonably good clinical recovery still have an approximately ten times greater risk of dying during the next 3 to 4 years than the average population of comparable age.

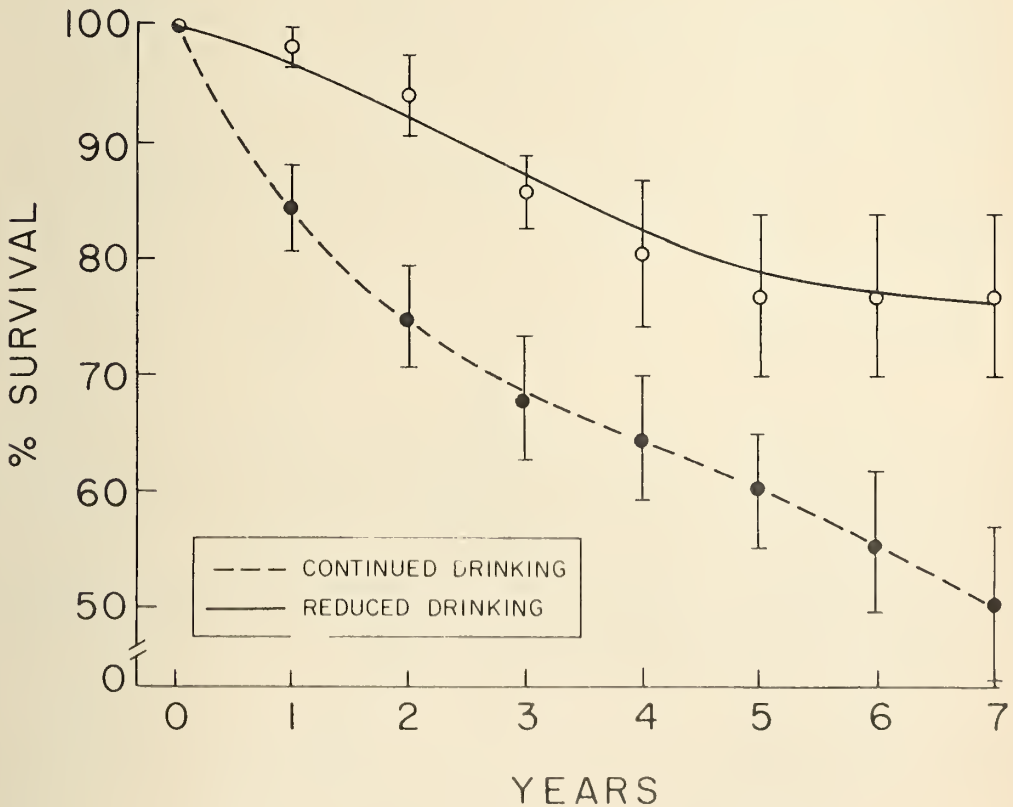


Figure 1. Survival rates from liver biopsy of 59 patients who either stopped drinking or demonstrated clearcut changes in their living habits as evidence for meaningful reduction of drinking compared to 98 patients who apparently continued to drink.

Among those individuals who continue their previous excessive drinking habits, the increased mortality rate remains unchanged after the 4th year of follow up in contrast to those individuals who stopped drinking and either remained abstinent or demonstrated a meaningful and significant reduction of their previous drinking habits (social drinking) (Figure 1). The consensus that continued excessive drinking is associated with increased mortality rate was based on studies performed in Atlanta, Georgia, New Haven, Connecticut and London, England. In contrast with these observations a recent study in Boston, Massachusetts reported that drinking did not affect the mortality rate of cirrhotic patients with esophageal varices (Table VI). However, one may wonder whether in the Boston study the number of patients who survived 5 to 6 years is sufficiently large to provide meaningful and biologically significant information and how the selection of the patients affected their mortality rate.

TABLE VI
5 YEAR SURVIVAL OF ALCOHOLIC LIVER DISEASE

	No.	Drinking		Entire group %
		Excessive %	Stop/Mild %	
London	123	34	69	50
New Haven (cirrhosis)	283	41	63	48
Boston (varices)	146	42	61	55
Atlanta (alcoholic hepatitis)	164	60	77	63
Atlanta (cirrhosis)	46	—	—	61

The prognosis is worse in alcoholic hepatitis in those who have cirrhosis or ascites (Figures 2 and 3). However, the presence of alcoholic hyalin or steatosis did not affect the survival rate. Although jaundice and elevated serum bilirubin were associated with increased mortality rates after the recovery from the acute illness, the elevated serum bilirubin had no independent prognostic value when it was corrected for the independent effect of ascites. None of the commonly used liver function tests had any prognostic value other than the prothrombin time referred to above.

THE THERAPY OF ACUTE ALCOHOLIC LIVER DISEASE

The cardinal principles of the treatment of alcoholic hepatitis are complete abstinence from alcoholic beverages, replacement of nutritional deficiencies, high protein, nutritious diet and bed rest. Although these therapeutic principles are generally accepted worldwide,

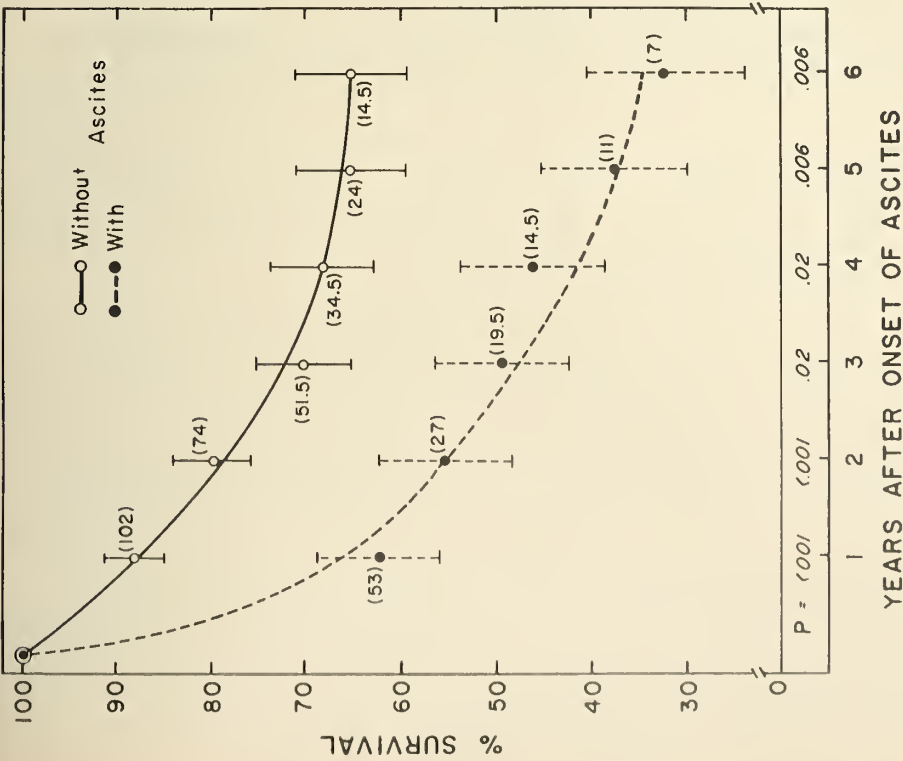


Figure 2. Survival rates from their first liver biopsy of 56 patients with ascites are compared with 108 patients without ascites.

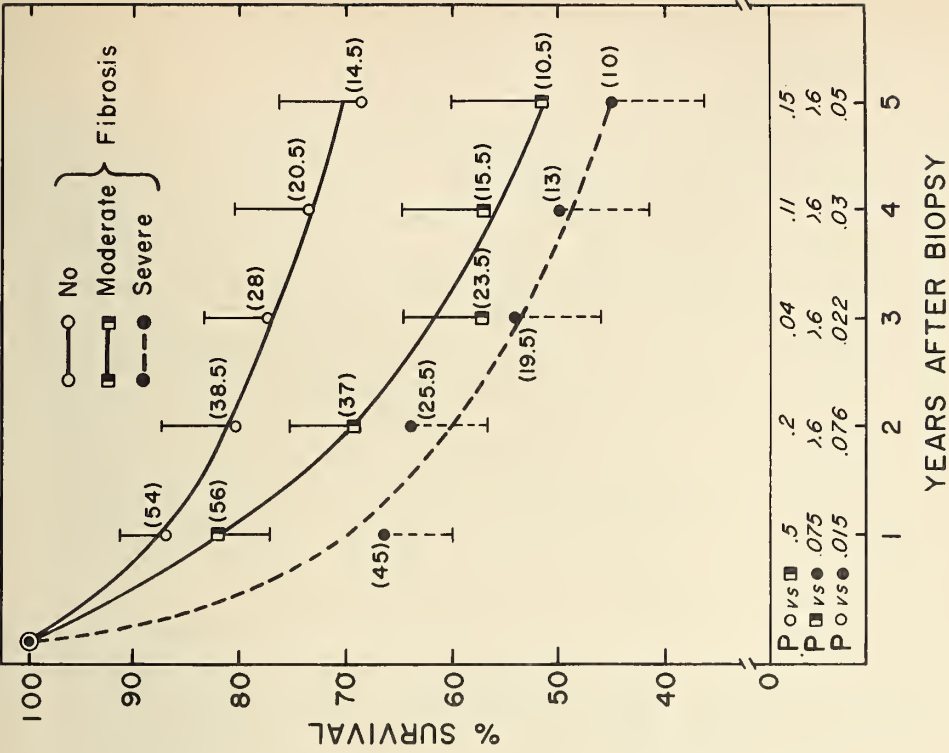


Figure 3. Survival rates from their first liver biopsy of 164 patients according to the severity of fibrosis. Vertical lines are \pm standard error. Patients were divided into three groups: 1.0 = no fibrosis, 2. □ = moderate fibrosis and 3. ● septum formation or cirrhosis.

it is not known how each specifically affects the course of the disease; nor has any of these principles withstood rigorous testing. Nevertheless, these are still the currently acceptable mode of treatment.

1. *Drinking.* — Although volunteers who consumed increasingly large amounts of ethanol developed progressively severe histologic lesions in the liver, drinking did not prevent their recovery from alcoholic hepatitis. However, the rate of recovery apparently increased after the discontinuation of the administration of ethanol-containing formula which also had an adequate protein and calorie intake. It is noteworthy that all these experiments of alcohol consumption and recovery from alcoholic liver disease are performed on the patient who is able to consume large amounts of food mostly in liquid formula. Whether complete abstinence is or is not essential for the recovery of alcoholic liver disease is an academic question. Many of the patients who develop alcoholic liver disease are also manifesting the disease syndrome of alcoholism. Abstinence in these patients is essential for more than one reason. Nevertheless, it is not clear at all that complete abstinence improves the morbidity and mortality in those patients who completely recovered from alcoholic hepatitis and who do not have the psychological problems of alcoholism. Whether these people may return to a normal "social" drinking pattern without undue hazard has not been disputed by prolonged observation.

2. *Nutritional deficiency.* — It is common practice to administer additional vitamins, particularly folic acid, to patients with alcoholic hepatitis. Despite claims, there is no evidence that either folic acid or the other vitamins have any significant effect on the process of recovery from alcoholic hepatitis beyond replacing vitamin deficiencies.

3. *Diet.* — For more than half a century, it became customary to prescribe high protein and high calorie diets to patients with liver disease of any kind. Often these diets are restricted in fat content which make them unpalatable. Patients with alcoholic hepatitis usually have poor appetites and, regardless of the physician's orders, the amount of food they consume is quite limited. When the usual hospital type food is changed to a good tasting, well flavored liquid formula, the total calorie intake, as a rule, increases. It is important to provide the patient with a sufficient number of carbohydrate calories during periods of anorexia to reduce maximally endogenous protein catabolism and gluconeogenesis. In some patients, forced feeding has to be resorted to by the use of nasogastric tube. The possible use of intravenous hyperalimentation has not been evaluated and one would have to exercise a great deal of caution to provide large amounts of intravenous nutrients to a damaged liver. Hyperosmolarity, hyperammonemia and hyperaminoacidemia may be an excessive hazard in these patients.

4. *Corticosteroids* — There has been a general consensus in France, Britain and the United States that the short term (4 to 14 days) corticosteroid therapy can improve, at least transiently, the appetite of the patient with alcoholic hepatitis. There is no other documented beneficial effect of corticosteroids that is specifically related to the course of alcoholic hepatitis. Several prospective double blind control studies clearly demonstrate the lack of any beneficial effect attributable to corticosteroids whether the diagnosis is made by biopsy or without biopsy if contraindicated. The claim that a specific group of middle-aged women with cirrhosis and ascites, as well as alcoholic hepatitis and transient encephalopathy, has benefited from corticosteroid therapy, has not been confirmed.

5. *Prognosis of the morphologic damage due to alcoholic hepatitis* — Serial biopsies of patients with alcoholic hepatitis have been studied in our institution (Table VII). The liver was examined on 188 occasions histologically in 61 patients whose first liver biopsy showed no evidence of cirrhosis or septum formation. Of these, 23 (38%) developed cirrhosis on an average follow-up of 2.8 years. In two of these patients the progression of

TABLE VII

LIVER HISTOLOGY AFTER ALCOHOLIC HEPATITIS

Lesion on last exam.	No. of patients	Years of follow-up \pm S.D.
Cirrhosis*	15	3.0 \pm 1.9
Cirrhosis**	23	2.8 \pm 1.5
Alcoholic hepatitis without cirrhosis	32	3.6 \pm 2.3
Normal	6	3.9 \pm 3

*Cirrhosis was present on the first biopsy.

**Cirrhosis developed in the interval.

cirrhosis was demonstrated on serial biopsies despite their abstinence during this period. In contrast, thirty-two (52%) continued to drink and had persistent evidence of alcoholic hepatitis during an average follow-up period of 3.6 years; none developed cirrhosis or septum formation. The remaining 6 (10%) who abstained, recovered normal hepatic architecture. It is important to note that recovery (both morphological and clinical) occurred not only in those patients with a clinically and histologically mild alcoholic hepatitis but also in those with a clinically and histologically severe hepatitis.

In contrast to the 32 patients with chronic alcoholic hepatitis in whom cirrhosis did not develop despite continued drinking, in 2 of the 23 patients who developed cirrhosis, its progression was demonstrated on serial biopsies despite their abstinence during this period.

A group of 16 patients with biopsy-proven alcoholic hepatitis but no significant fibrosis or septum formation, volunteered for a prospective study. Eleven of the 16 patients abstained during the one year follow-up period. Serial biopsies were obtained at 1, 4, 7 and 12 months after hospitalization with typical acute alcoholic hepatitis. All were seen by a single physician two to four times a month to establish a close rapport. In all patients there was a marked improvement of fatty infiltration after 4-5 weeks of intensive hospital therapy; however, the second biopsy showed a much more vigorous cellular inflammatory reaction in the areas of necrosis than the first biopsy which was obtained shortly after a period of excessive drinking. While the first follow-up biopsy showed a preponderance of PMN-leukocytes, the biopsies at 4 and 7 months showed increasing proportions of lymphocytes, macrophages and plasma cells in the cellular inflammatory reaction.

Five of sixteen patients who returned to drinking showed persistent alcoholic hepatitis in each of the follow-up biopsies; 2 of these 5 developed cirrhosis within the succeeding 18 months. Two of the eleven who abstained during the follow-up period died from causes unrelated to liver disease. An autopsy performed within 7 months showed alcoholic hepatitis. Of the remaining 6 patients who abstained, 4 improved; but 2 developed active septum formation and cirrhosis within 14 months.

TABLE VIII

DIRECTION OF CHANGE IN SEVERITY OF LESIONS
BETWEEN FIRST AND LAST EXAMINATION

Lesion	Severity of Lesion		
	Increased (↑)	Unchanged (=)	Decreased (↓)
Alcoholic hepatitis	20	28	28
Fibrosis	32	41	3
Steatosis	12	20	44

TABLE IX

X² TEST OF ASSOCIATION BETWEEN THE DIRECTION
OF CHANGE IN THE SEVERITY OF FIBROSIS AND
THAT OF ALCOHOLIC HEPATITIS

Alcoholic Hepatitis	Fibrosis			Total	
	↓	=	↑		
↑	14	6	0	20	X ² = 16.062
=	12	16	0	28	df ^a = 4
↓	6	19	3	28	P < 0.01
Total	32	41	3	76	

^aDegrees of freedom

It is noteworthy that either alcoholic hepatitis progressed to cirrhosis within 18 months on serial biopsies, or cirrhosis was seen on the first follow-up biopsy. In contrast, 20 patients who did not develop cirrhosis during the first 18 months, continued to drink. Serial biopsies showed evidence of alcoholic hepatitis without progression to cirrhosis up to 8 years. The progression of hepatocellular necrosis was associated with progressive fibrosis (Tables VIII and IX).

SUMMARY

The frequency of alcoholic liver disease is correlated with the per capita consumption of alcohol in various countries. The pathogenesis of alcoholic hepatitis usually requires prolonged and excessive drinking, although it may develop in some patients in less than a year. Although drinking is essential, drunkenness is not. Genetic factors probably determine the susceptibility to ethanol-induced liver disease. Alcoholic hepatitis is, but alcoholic fatty liver is not, the cause of alcoholic cirrhosis. The diagnosis of alcoholic hepatitis

depends on liver biopsy. The most common findings are: (a) subjective: recent excessive drinking, anorexia, nausea, abdominal pain and weight loss; (b) objective: hepatomegaly, jaundice, fever, ascites and splenomegaly; leukocytosis, anemia, SGOT < 500, SAP, hyperbilirubinemia, low albumin, elevated gamma globulin and IgA. Mortality rate is about 7% if prothrombin time is close to normal and is about 40% if it is prolonged. The long term survival rate is significantly affected by drinking, prothrombin time, ascites and by cirrhosis. None of the other clinical, biochemical or morphologic findings have a significant prognostic implication. The principle factors in the therapy are abstinence, adequate diet, and bed rest. However, none of these therapeutic principles has withstood the scrutiny of an adequate clinical trial. Corticosteroids do not improve the alcoholic hepatitis condition. Alcoholic hepatitis, even under optimal conditions, persists for at least 4 months if it is mild, or up to a year if the lesion is more severe. Prognosis of the morphologic lesion of alcoholic hepatitis depends on the severity of the lesion on the original biopsy. A third of the patients developed cirrhosis on serial biopsy. If cirrhosis develops, it is likely to occur within 1½ years. Continued drinking is associated with the persistence of alcoholic hepatitis without progression of cirrhosis in half the patients. Recovery of a normal liver architecture is possible only in those who abstain from drinking.

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The Mallory Body in the Pathogenesis of Alcoholic Liver Disease

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INTRODUCTION

Overview

The pathogenesis of alcoholic hepatitis and alcoholic cirrhosis has eluded investigators. No experimental model duplicating alcoholic hepatitis has been found although facsimiles have been reported (Porta, Hartroft, and de la Iglesia, 1965; Takeuchi, Takada, Kato, Hasumura, Ikegami and Matsuda, 1971 and Chey, Kosay, Siplet and Lorber, 1971).

One of the hallmarks of alcoholic hepatitis which has been missing from proposed experimental models of alcoholic hepatitis, is the complex consisting of Mallory's hyaline bodies (MB) and focal infiltrations of polymorphonuclear (PMN) leukocytes (Schaffner and Popper, 1970). The failure to experimentally induce this complex has prevented advances in the understanding of alcoholic hepatitis. It has forced investigators to limit their studies to the human liver. This limitation severely restricts the investigation, because the human liver is not available for experimental manipulations. Only needle biopsies or post mortem material can be used. Needle biopsies are inadequate because of the small amount of material available for study and because sampling errors are possible owing to the focal nature of the MB-PMN complex. Post mortem material is inadequate because the autolytic process introduces unknown variables including protein denaturation and degradation. These restrictions leave investigators with unsatisfactory compromises in methodology. These compromises have no doubt discouraged many capable scientists from investigating the problem, and explain why investigations done thus far have been limited to clinical, histochemical and electron microscopy studies. Recently, it was possible to isolate Mallory bodies in a purified fraction (French, Ihrig and Norum, 1972). Electron microscopy studies of this fraction revealed that the MB was

composed of a microfilament-like skeleton (French, Wiggers, French and Carr, 1973). This finding has implications regarding the basic nature of the MB. A foundation is laid for making a hypothesis how MBs are formed and how MB formation may lead to failure of hepatocellular function.

History

The Mallory body was first defined by Mallory (1911) as an irregular, coarse, hyaline meshwork (Fig. 1) which stains deeply with eosin. He noted that after the hyaline change had reached a certain degree of intensity, the affected liver cells were surrounded (Fig. 2) and invaded by numerous polymorphonuclear (Fig. 3) or endothelial (Fig. 1) leukocytes.

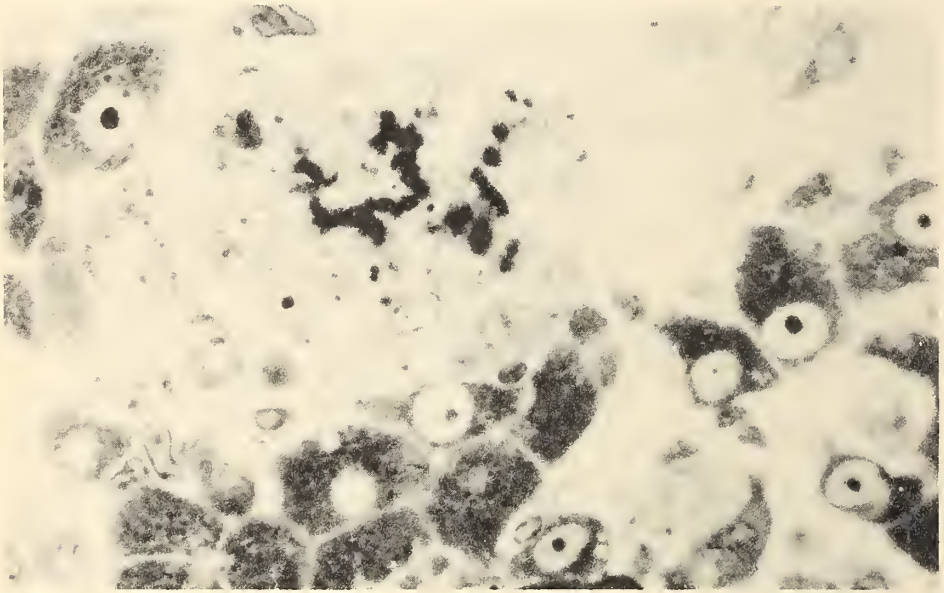


Figure 1. *Liver biopsy showing a Mallory body in a case of alcoholic hepatitis. The rope-like character is evident. The body is partially surrounded by macrophages. Toluidine blue.*

The affected cells and the nuclei within them are usually swollen (Fig. 2). Mallory noted the association of the hyaline and leukocytic infiltrate with regeneration of liver cells, proliferation of connective tissue, and focal bile stasis. This constellation of findings is the morphological component of so-called alcoholic hepatitis (Phillips and Davidson, 1954; Schaffner and Popper, 1970; Jewell, Medline and Medline, 1971; Christoffersen, 1972; Gregory and Levi, 1972; and Lesesne and Fallon, 1973). Mallory postulated that the fibroblastic proliferation that leads to cirrhosis was the result of the leukocytic infiltration. Recently, Schaffner and Popper (1970) have restated this hypothesis, but they suggested that the hyaline acts as an irritant to stimulate fibrosis directly. However, since MBs are sometimes absent in alcoholic hepatitis (Christoffersen and Juhl, 1971; Lischer, Alexander and Galambos, 1971; and Harinasuta and Zimmerman, 1971) it is unlikely that they are essential for the development of alcoholic cirrhosis. When MBs are present, however, the clinical and histologic features of alcoholic hepatitis tend to be more severe (Gregory and Levi, 1972; and Harinasuta and Zimmerman, 1971 and Galambos, 1973).

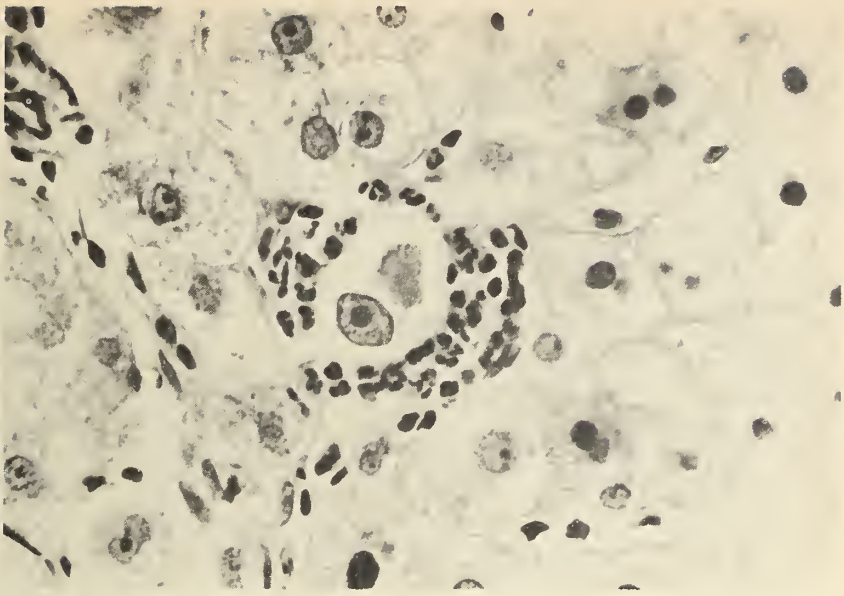


Figure 2. Liver biopsy showing the Mallory body-PMN complex. The liver cell containing the body has abundant clear cytoplasm and an enlarged nucleus with a prominent nucleolus. A ring of PMNs surrounds the involved cell. Surrounding liver cells are not involved. Hematoxylin and eosin.

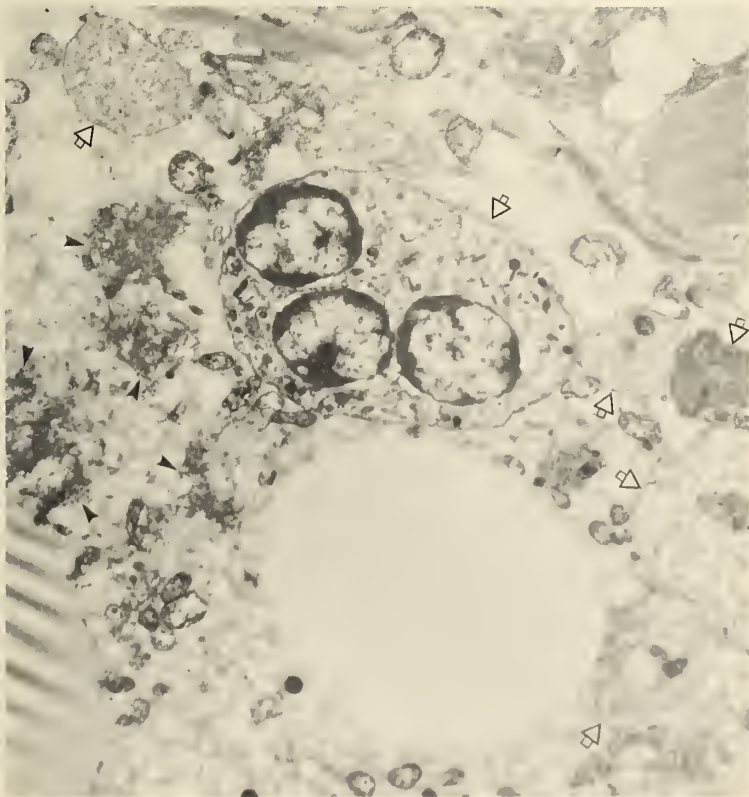


Figure 3. Liver biopsy showing a portion of a fatty liver cell which contains MBs (solid arrow heads). Leukocytes (PMNs) are seen within the cytosol of the hepatocyte (open arrows). Often only the pseudopod portion of the PMNs is seen. EM, 9760X.

The importance of the MB-PMN complex (Figs. 2 and 3) as a hallmark of alcoholic liver disease has been emphasized by numerous investigators (Huber and Englehart, 1967; Schaffner and Popper, 1970; Christoffersen, 1972). Mallory (1911) stated that leukocytes disposed of the hyaline by phagocytosis. Schaffner and Popper (1970) illustrated the attack of MBs by PMNs and stated that they found MBs within macrophages and leukocytes. Many others have illustrated PMNs surrounding and/or invading hepatocytes containing MBs. In Figure 3, numerous pseudopodia of leukocytes can be seen within the cytoplasm of a hepatocyte containing MBs. The plasma membrane of the involved hepatocyte is missing so that the plasma membranes of the leukocytes are in direct contact with the cytosol and organelles of the liver cell. We have noted similar changes to involve liver cells which did not contain Mallory bodies. In these studies, serial sections were examined by electron microscopy. Organelles such as mitochondria and MBs appeared to fit into recesses of the plasma membrane of the leukocytes. However, phagocytic vacuoles were not found to contain phagocytized material that resembled MBs or mitochondria. Although it has been postulated that MBs are chemotactic for leukocytes (Schaffner and Popper, 1970; French, 1971; Christoffersen, 1972); the mechanism of the chemotaxis has never been investigated. Cellular immune mechanisms could be involved (Sorrel and Leevy, 1972). The fact that alcoholic hepatitis progresses to cirrhosis during the recovery period in the absence of alcohol ingestion (Helman, Temko, Nye and Fallon, 1971; and Galambos, 1973) is consistent with an autoimmune mechanism.

Mallory bodies are not unique to alcoholic liver disease (Gerber, Orr, Denk, Schaffner and Popper, 1973), nor do they occur exclusively in liver tissue (Kuhn and Kuo, 1973). Gerber *et al.* (1973) observed MBs in alcoholic liver disease, primary biliary cirrhosis, extrahepatic biliary obstruction, chronic aggressive hepatitis, "cryptogenic" cirrhosis, fatal Wilson's disease and Indian childhood cirrhosis. Gerber *et al.* (1973) confirmed the presence of MBs by electron microscopy only in the case of alcoholic liver disease and primary biliary cirrhosis. This leaves in doubt whether extrahepatic biliary obstruction and the other conditions studied by Gerber, *et al.* (1973) really are associated with MBs since structures such as megamitochondria have been mistaken for MBs using the light microscope (Iseri and Gottlieb, 1971). Even Mallory himself apparently had difficulty identifying MBs by light microscopy (Mallory, 1933). That MBs must satisfy electron microscopy criteria before positive identification can be made, cannot be over-emphasized. Using these criteria MBs have been documented in alcoholic liver disease (Biava, 1964; Flax and Tisdale, 1964; Smuckler, 1968; Feldman, Oudea, Molas, Domart-Oudea, and Fauvert, 1970; Soga, Terada, Kasukawa and Koizumi, 1970; Iseri and Gottlieb, 1971; Albukerk and Duffy, 1972; French, *et al.*, 1972; Ma, 1972; Gerber *et al.*, 1973; Yokoo, Minick, Batti, and Kent, 1972; and Horvath, Kovacs and Ross, 1973), primary biliary cirrhosis (Gerber *et al.*, 1973; and Monroe, French and Zamboni, 1973), Indian childhood cirrhosis (Roy, Ramalingaswami and Nayak, 1971); hepatoma (Keeley, Iseri and Gottlieb, 1972) and asbestosis (Kuhn and Kuo, 1973).

The electron microscopy studies of Biava (1964) and Yokoo, *et al.* (1972) have contributed the most to the understanding of the MB ultrastructure. Biava (1964) illustrated the fibrillary tubular character of the Mallory body but he mistook the fibrils for fragmented endoplasmic reticulum. Yokoo, *et al.*, (1972) subclassified Mallory bodies into three types. Type I is characterized by filaments which run parallel to each other (Figs. 4-6). These filaments have a zig-zag or "decorated" appearance. They interconnect with each other in an irregular manner. When they are cut across they appear as clusters of black dots (Fig. 7). Their thickness is variable. According to Yokoo *et al.* (1972) they averaged 14.1 nm in diameter. Type II filaments (Fig. 4, 7, and 8), the most common

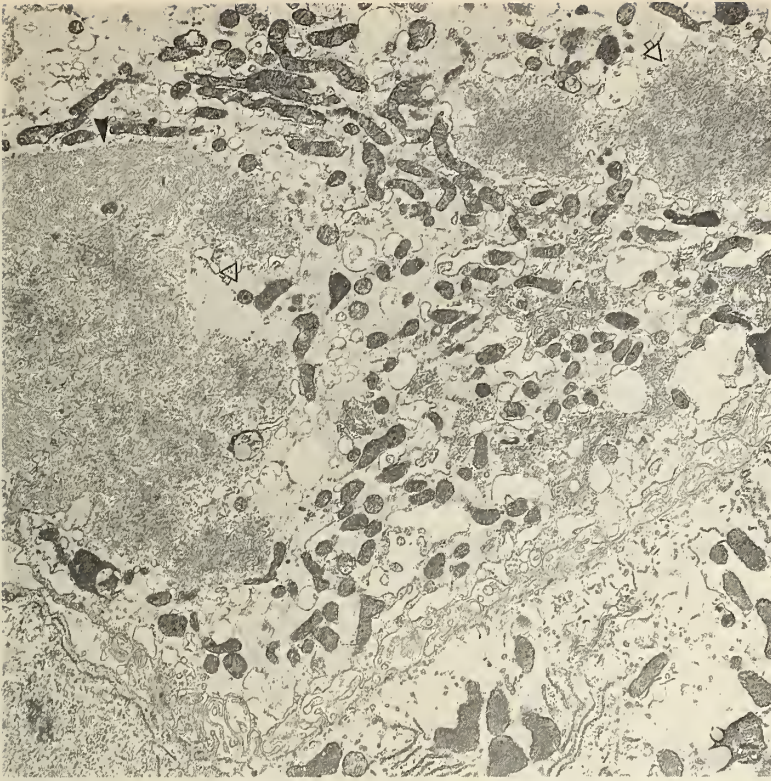


Figure 4. Liver biopsy showing type I (solid arrow head) and type II MBs (open arrows). Notice the disarray of intracellular organelles. Numerous Golgi are located mid-right portion of the cell, EM, 10,300X.

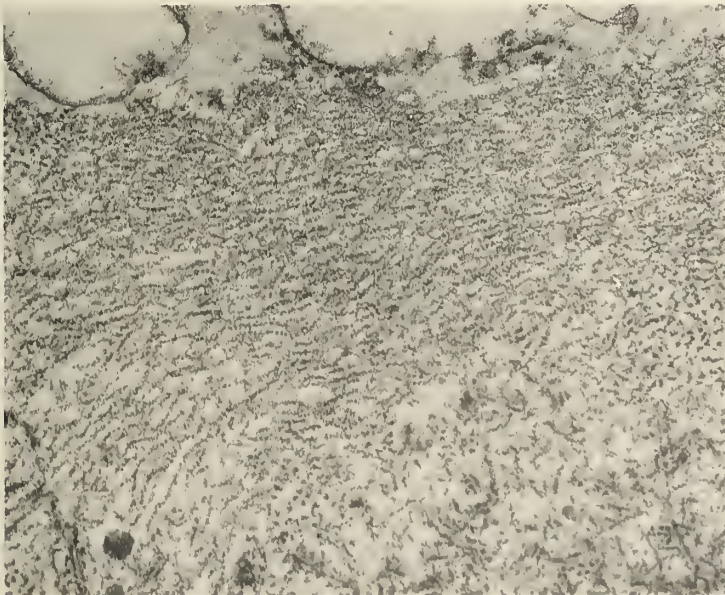


Figure 5. Liver biopsy showing the type I MB filaments. The filaments run parallel and interconnect. Note the beaded appearance of the filaments and how they appear as dots on cross section. The type I filaments merge with type II filaments at the bottom. EM, 59,500X.

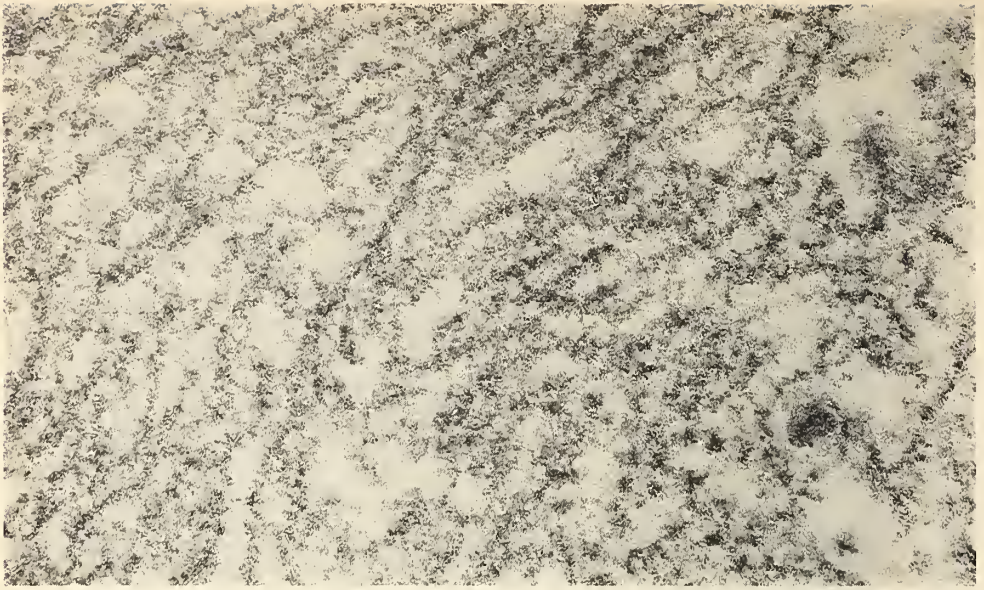


Figure 6. Type I MB filaments as seen in Fig. 5. Note that the beaded appearance is probably due to "decoration" by material attached to the filaments. This finding is similar to HMM binding to actin-like microfilaments (Figs. 17 & 18). Interconnections are numerous. EM, 165,200X.

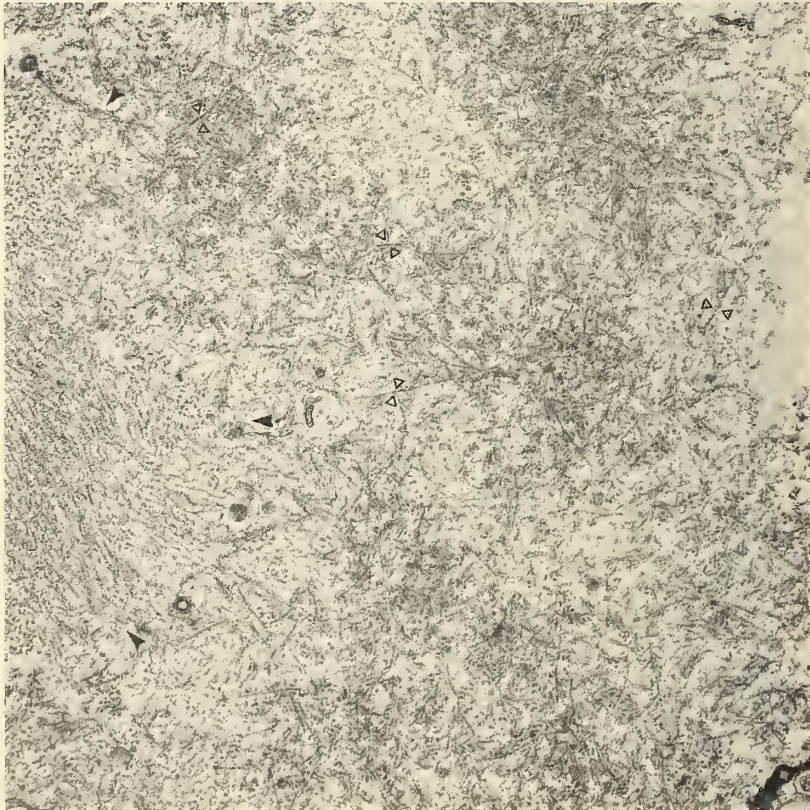


Figure 7. Type I MB filaments as seen in Fig. 5 (solid arrowheads to the left) and type II filaments in the middle and right. Branching points of the type II filaments are seen between the open triangles. EM, 36,300X.

type, consist of randomly oriented fibrils averaging 15.2 nm thick. Wiggers, French, French and Carr (1973) noted that these filaments branch (Fig. 7) and have a tubular appearance (Fig. 8). When the tubular structures were measured, excluding material which appeared to bind and thicken the filaments, they averaged 8 nm thick. Others have measured the filaments at 8-10 nm (Biava, 1964), 15-18 nm (Albukerk and Duffy, 1972), 15-17 nm (Smuckler, 1968), 9-14 nm (Kuhn and Kuo, 1973) and 15-20 nm (Gerber, *et al.*, 1973). The third variant (Type III) Mallory bodies described by Yokoo, *et al.* (1972) was composed of granular or homogeneous electron dense material. Filaments were sometimes seen within the material and around the edge. Figures 9 and 10 in Kuhn and Kuo's paper (1973) illustrate Type II and III MBs in continuity with each other. Type II surrounded Type III. The relationship of Type I and II in the liver cells is not regular (see Figs. 4, 5 and 7). The important concept to emerge from these studies is the idea that Mallory bodies may be derived from filaments normally involved in the contractile system of liver cells (Yokoo, *et al.*, 1972).

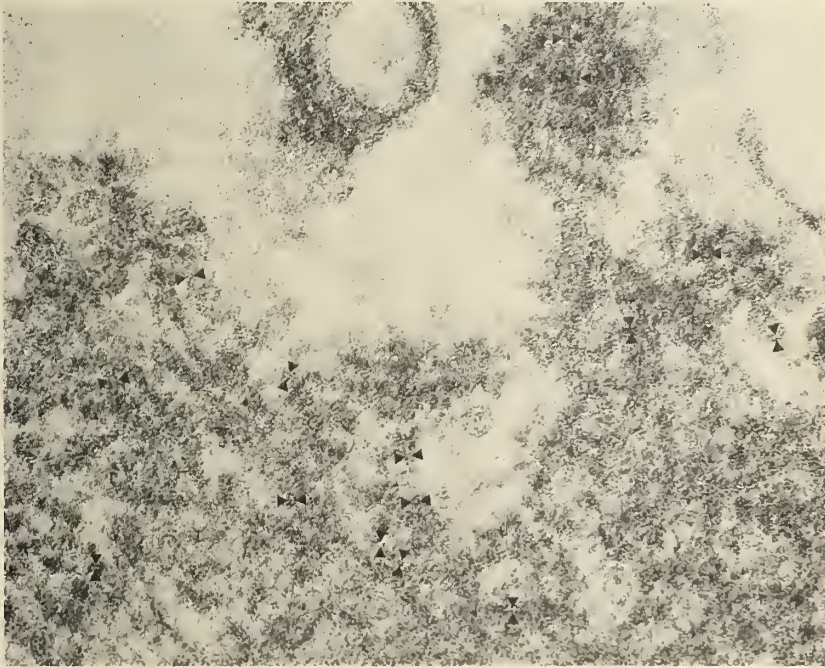


Figure 8. Liver biopsy showing tubularity of the MB filaments (between solid triangles). These filaments average 8nm in diameter EM, 195,000X.

The origin of Mallory bodies has not been determined. There have been a number of speculations regarding the origin of MBs based on which organelles were found next to them. Many investigators have emphasized the close association of MBs to granular endoplasmic reticulum and free ribosomes (Biava, 1964; Roy, *et al.*, 1971; Ma, 1972; Monroe, *et al.*, 1973; and Horvath, *et al.*, 1973). Others have not found a relationship between MBs and any cell organelle (Smuckler, 1968; Yokoo, *et al.*, 1972; Albukerk and Duffy, 1972; Gerber, *et al.*, 1973). Flax and Tisdale (1964) and Soga *et al.* (1970) felt that the MBs were probably derived from focal cytoplasmic degradation which included lysosomes. Keeley *et al.*, (1972) emphasized the association of microbodies with MBs. It seems clear from ultrastructural studies that there is no consensus regarding the origin of MBs. More sophisticated techniques will have to be employed before the problem can be

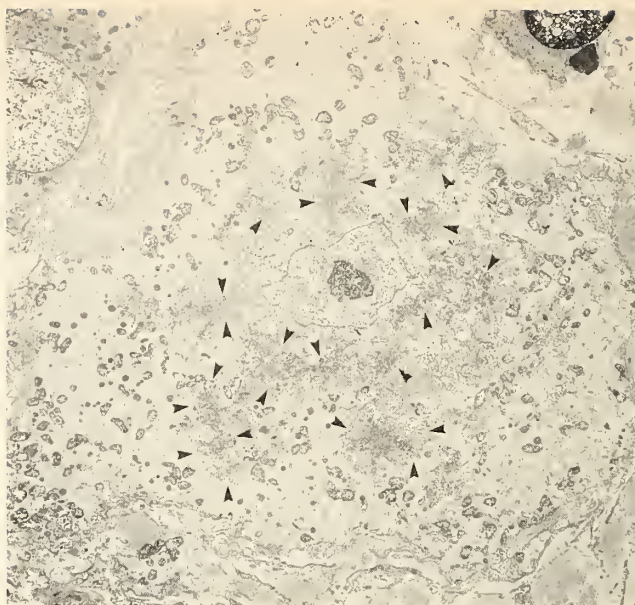


Figure 9. Liver biopsy showing a fat-laden liver cell with numerous small foci of MBs (solid arrow heads). The MBs are surrounded by granular endoplasmic reticulum (GER). This liver cell is enlarged and is surrounded by bundles of collagen fibers. A macrophage containing phagosomes is present at the upper right and a more normal liver cell is seen to the upper left. EM, 4046X.

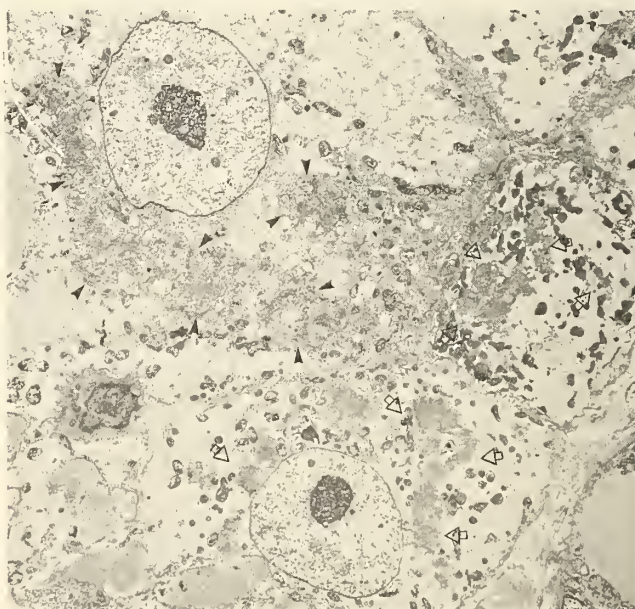


Figure 10. Liver biopsy showing MBs in different intracellular environments. The hepatocyte at the top contains MBs (solid arrow heads) within areas of GER in a cytosol which is otherwise filled with small fat spheres similar to Fig. 9. The hepatocytes to the right and below show MBs (open arrows) suspended in an amorphous cytosol which appears devoid of GER. Glycogen is present in the cell at top but absent in the cells to the right and below. A monocyte is seen invading the cytosol of a fatty hepatocyte to the left. Note the maldistribution and distortion of the mitochondria in the hepatocyte to the right; EM, 4320X.

solved. Nevertheless, our working hypothesis is that MB protein is synthesized by the ribosomes, either attached or free. We have noticed (Monroe, *et al.*, 1973) that the smaller aggregates of MB filaments are consistently associated with ribosomes and polyribosomes (Fig. 9 and 10). When the aggregates enlarge the association with ribosomes is usually focal or non-existent (Figs. 10 and 11). Often larger MBs lie suspended in amorphous cytosol which is relatively free of organelles (Fig. 10 and 11). Others have postulated that MBs are derived from *de novo* synthesis (Yokoo *et al.*, 1972; Albukerk and Duffy, 1972; Horvath *et al.*, 1973; and Keely *et al.*, 1973). It seems reasonable to postulate, therefore, that MB protein is first synthesized by aggregates of ribosomes. However, instead of dispersing throughout the cytoplasm, the newly synthesized protein assembles in filament form (Albukerk and Duffy, 1972, and Horvath, *et al.*, 1973) owing to an abnormality which is as yet not known. As the mass of filaments accumulate, the original aggregate of ribosomes disperses or becomes dwarfed by the growing MB so that the chance of finding associated ribosomes decreases as the MB enlarges. Unfortunately, this hypothesis cannot be tested until an animal model is available for experimental induction of MBs.

The fate of Mallory bodies is also unknown. The evidence for disposal by phagocytosis or digestion by PMNs has already been discussed. Certainly, clinical follow-up studies show that MBs may decrease in number or disappear during abstinence (Green, Mistilis and Schiff, 1963; Helman *et al.*, 1971 and Galambos, 1972). Albukerk and Duffy (1972) noted that filaments less than 10 nm thick and over 16 nm thick disappeared in MBs from patients who abstained from alcohol for 11 or more days. The fibrils appeared sparsely distributed. This finding suggests that the fibrils may be lost through dispersion. They noted that increased numbers of lysosomes and autophagocytic vacuoles were among the poorly preserved fibrils. Albukerk and Duffy postulated that MBs disappear through autolytic digestion and autophagocytosis. Thus, there are three possible fates for the MB. If the liver cell dies, digestion through leukocytic activity may occur. If the liver cell survives, dispersion or autodigestion may result.

The chemical nature of Mallory bodies has been investigated histochemically. The results have been conflicting and inconclusive, probably because of the numerous problems inherent in the methodology. Certain conclusions, however, are tenable. For instance, it is likely that the MB is composed of a basic protein. Roque (1953), showed that MBs were digested by proteolytic enzymes, gave a positive reaction for arginine and showed maximum binding of acidic dyes at pH 1.8-2.2. The basic protein nature was confirmed by Norkin, Weitzel, Campagna-Pinto, MacDonald, and Mallory (1960), Becker (1961) and Norkin and Campagna-Pinto (1968). According to the histochemical findings of Lyon and Christoffersen (1971), the isoelectric point of MB protein is between pH 5 and 6. Norkin *et al.* (1960) showed that the Mallory body was resistant to solvents such as detergents, and lipid solvents. It resisted extraction at various concentrations of salts and H^+ and required prolonged digestion with trypsin or pepsin before it disappeared. This feature of insolubility aids the isolation of MB from the liver (French, *et al.*, 1972). It is also an indication MBs form and accumulate as a result of their decreased solubility.

Further characterization of the chemical composition of Mallory bodies is difficult because of the conflicting results reported by various authors. Suffice it to say that some investigators have found histochemical evidence of carbohydrate (Roque, 1953; and Lyon and Christoffersen, 1971), and lipid (Becker, 1961; Norkin and Campagna-Pinto, 1968; Nayak, Sagreiya, and Ramalingaswami, 1969; and Lyon and Christoffersen, 1971) in MBs. There is some disagreement regarding the activity of various enzymes in MBs (Norkin, *et al.*, 1960; French, 1960; Nayak, *et al.*, 1969; Lyon and Christoffersen, 1971;

and Ma, 1972). Some of this disagreement may be due to the fact that autopsy livers were utilized in some studies and liver biopsies were used in others. Also, criteria used to positively identify MBs may have differed in the various studies. In addition, incorporation of organelles such as mitochondria and lysosomes could introduce another variable. For instance, Lyon and Christoffersen (1971) concluded that MBs contained chemical evidence suggesting the presence of decomposed mitochondria, lysosomes, ribosomes and endoplasmic reticulum. There is a need then to be able to separate the elements which become incorporated into the MB from fibrils of which the MB is primarily composed. This separation implies visualization of the MBs utilizing histochemical techniques adapted for evaluation by electron microscopy. Ma (1972) has successfully employed these techniques. He showed that the MBs did not react for acid phosphatase, nucleoside diphosphatase or for cytochromes. Adjacent mitochondrial cristae reacted positively for cytochromes. This does not support a mitochondrial origin of MBs as was suggested by the histochemical studies of Minaker and Porta (1967).

NEW OBSERVATIONS

The Microfilament Skeleton of Mallory Bodies

The filament skeleton which constitutes the essence of type II MBs as viewed in liver biopsies has already been described (Figs. 5, 7, 8, and 11). When the ultrastructure of *in situ* MBs from three cases of alcoholic hepatitis was compared with MBs isolated from

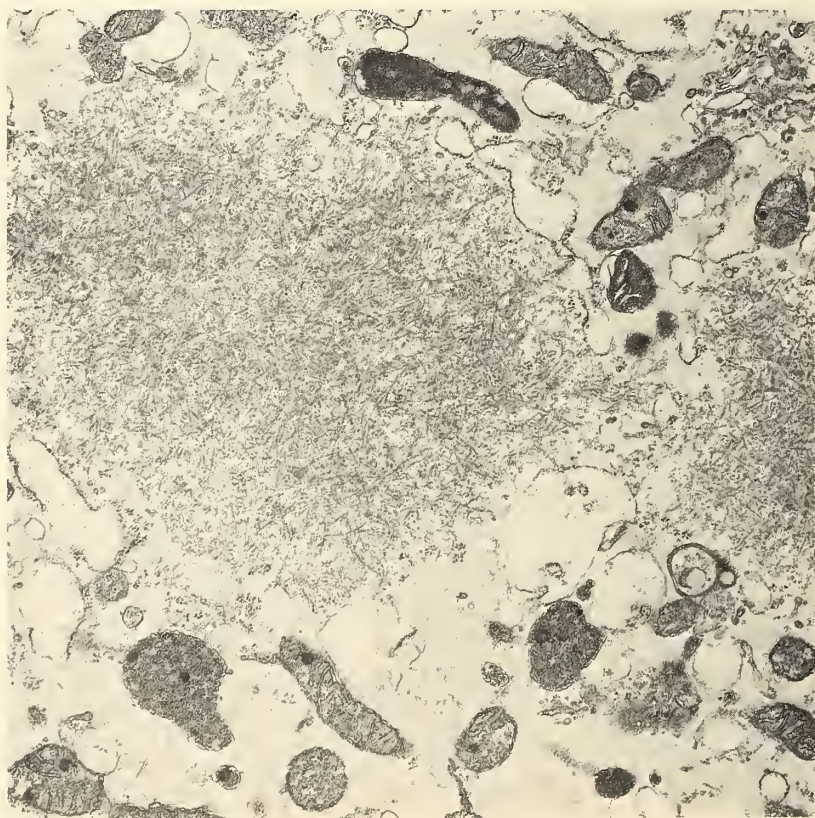


Figure 11. Liver biopsy showing type II MBs. The filamentous nature of the MBs can be seen against a background of amorphous cytosol. EM, 28,600X.



Figure 12. MB filaments isolated by discontinuous density gradient centrifugation from an autopsied patient who died with alcoholic hepatitis. Notice the branching of filaments (solid arrow heads) and the bridging of filaments to form rectangles (between open triangles). Filaments cut on end appear as dot-like densities. EM, 150,600X.

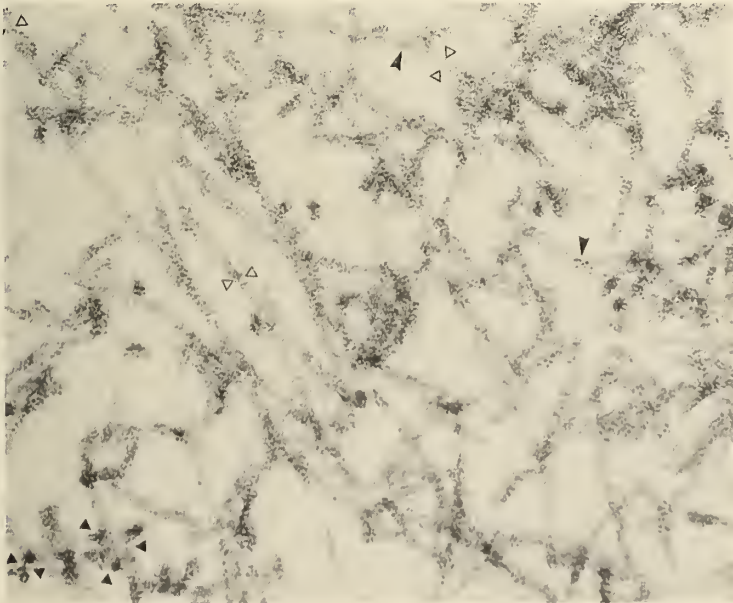


Figure 13. Isolated MB filaments showing branching (solid arrow heads) and tubularity (between open triangles). Cross sections of the filaments show irregular lucent areas (between solid triangles). These filaments average 8nm in diameter. EM, 231,000X.

three autopsied cases of alcoholic hepatitis (using the isolation method of French, *et al.*, 1972), the filament character of the MB became more obvious (Figs. 12 and 13). The branching and tubular character of the isolated filaments bore a close resemblance to *in situ* filaments. In both situations, the filaments were long and straight or slightly curved. High power magnification of the filaments showed a granularity of the tubule walls (Fig. 13). This granularity suggests the existence of a sub-unit molecule with which the tubules are assembled. This is analagous to the subunits observed in neurofilaments and glial filaments which also exhibit tubularity (Wuerker, 1970; and Schmitt, 1968). The granularity observed could conceivably account for the beading of the *in situ* MB filaments described by others (Albukerk and Duffy, 1972). The beaded appearance of the MB filaments described by Gerber, *et al.*, (1973) may be due to material bound to the filaments since it gave a periodicity of 25 to 35 nm. The beading described by Yokoo, *et al.*, (1972) was observed in type I Mallory body filaments. Such "beading" can be easily seen in Figures 5 and 6. It resembles the decoration of actin filaments by heavy meromyosin binding (Pollard and Kern, 1973; and Chang and Goldman, 1973). The lack of material bound to the isolated MB filaments as compared to the *in situ* filaments is probably due to the solubilization of such material during incubation of the isolated MBs in 5% deoxycholate (French, *et al.*, 1972).

When the MB filaments are seen in cross section (Figs. 12 and 13), they appear as electron dense rounded dots which resemble ribosomes. They have irregularly placed electron-lucent cores (Fig. 13). These structures were described by Biava (1964) in *in situ* MBs (see Biava's Fig. 29). Biava interpreted these structures to represent altered ribosomes which had lost ribonucleic acid. It seems more likely that they represent cross sections of filaments since they are sometimes seen cut tangentially and therefore have a slightly elongated appearance (Fig. 13). The average diameter of the *in situ* MB filaments was 8 nm. The average diameter of the isolated filaments was also 8 nm. Their branching and tubularity were also quite similar. It is likely, therefore, that isolated MBs are in fact derived from *in situ* MBs.

In order to guess the nature of Mallory body filaments, it is appropriate to ask the question: are there any filaments which occur in cells which are 8 nm thick and exhibit a branching tubular morphology? Yokoo, *et al.* (1973) described fine filaments (7.4 ± 2.1 nm) that resembled normal liver cell tonofilaments. These fine fibrils were seen in liver and ductular cells with or without associated MBs. They ran in parallel bundles. Neither branching nor tubularity of the fibrils was described. There are a rather large number of reports describing intracellular filaments of possible contractile nature in a variety of cells (for a review see Table I of a report by Newstead, 1971). The filaments described were often within the range of 6nm to 8nm thick but the combined branching and tubular appearance was not described. De Petris, Karlsbad and Pernis (1962) illustrated the branching character of microfilaments (4.6 nm in thickness) in monocytes. Carr (1972) described branching microfibrils (average 6 nm thick) in macrophages within human lymph nodes. Electron micrographs of numerous cell types have shown microfilaments arranged in a manner which mimics the architecture of the isolate type II Mallory body filaments. This pattern can be seen in human monocytes (Fig. 8, De Petris, *et al.*, 1962), in human heart myxoma cells (Figs. 16 and 17, Ferrans and Roberts, 1973), in altered human platelets and extracted thrombosthenin (Figs. 6, 7 and 14; Zucker-Franklin, 1969), in human granulosa and Sertoli cells (Fig. 2b, and c, Schuchner and Stickert, 1973), in human epidermal basal cells (Figs. 3 and 7, Wolff-Schreiner and Wolff, 1973), in cultured neuroblastoma cells which have been glycerinated and exposed to heavy meromyosin (Fig. 5, Chang and Goldman, 1973), and in isolated neurofibrils (Fig. 3,

Shelanski and Taylor, 1970). Thus, it would seem that the branching filament architecture of type II MB has its counterpart in numerous types of normal and altered cells. This observation is offered as important evidence that type II MB filaments are a pathologic expression of a normal structure within the liver cell.

Identification of Hepatic Contractile Protein

Given that the hypothesis of Yokoo, *et al.*, (1972) is correct, that MB filaments are derived from the hepatic contractile system, which component of this system could the MB filaments represent? Since the MB filaments measured 8nm thick, it is possible that they are thin filaments which bind heavy meromyosin and are actin-like. For instance, Pollard, Shelton, Weihing, and Korn (1970), Pollard and Korn (1972), Pollard (1973) and Pollard and Korn (1973) have shown that the thin filaments (6-7 nm thick) of *acanthamoeba* are composed of F-actin, similar to skeletal muscle actin. They used rigorous criteria for identification including functional properties, myosin interaction, physical properties and chemical composition. However, they pointed out that the simplest way to identify tentatively a filament as actin is to see if the filament will bind heavy meromyosin (HMM) to form characteristic arrow-heads as seen by electron microscopy. No other filament is known to bind HMM. If Mg-ATP or Mg-pyrophosphate dissociate the filament-HMM complex then it is reasonable to conclude that it is F-actin. Using this approach HMM binding filaments have been identified in muscle, fibroblasts, chondrogenic cells, nerve cells, and epithelial cells of the epidermis, intestine and trachea (Ishikawa, Bischoff and Holter, 1969). These authors showed that HMM did not bind to 10 nm filaments or the tonofilaments associated with desmosomes of epidermal cells, intestinal or tracheal epithelium. Chang and Goldman (1973) found HMM binding actin-like filaments in cultured neuroblastoma cells. Luduena and Wessells (1973) found that HMM bound two types of filaments in nerves and migratory cells. The first was a lattice network involved with cell extension and ruffling. The second type of filament occurred as a sheath of parallel filaments which ran along the long axis of cells and were involved in contraction. Behnke, Kristensen and Nielsen (1971) showed that actin-like microfilaments in platelets bound HMM. Allison, Davies and De Petris (1971) showed that actin-like filaments in macrophages bound HMM. Schroeder (1973) showed that contractile ring filaments involved in cytokinesis bound HMM. Spooner, Ash, Wrenn, Frater and Wessells (1973) found that HMM bound to actin-like filaments in embryonic tissues or cultures including salivary gland, lung, oviduct, glial cells and cardiac myoblasts and fibroblasts.

The liver cells have not been tested for the presence of filaments which bind HMM. Attempts to extract actin from adult liver failed to show a prominent actin-like band on acrylamide gel electrophoresis (Bray, 1972). Using a fluorescent labelled antibody to human platelet actomyosin, Becker and Nachman (1973) failed to localize contractile protein in liver cells. Presumptive evidence of actin in liver cells was reported by Gabbiani, Ryan, Lamelin, Visalli, Majno, Bouvier, Cruchaud and Lüscher (1973). Gabbiani, *et al.* (1973) used a serum auto-antibody to smooth muscle to locate actin-like material in liver cells but they did not study binding of the antibody to purified actin. Therefore, we decided to see if rat liver cells and isolated MB filaments would bind HMM. We also attempted to extract actin from adult rat liver.

Three rat livers and three rat parotids were glycerinated and then exposed to HMM. The liver and parotid tissue were obtained fresh on different days and each time the HMM

was prepared fresh to test the reproducibility of the results. The HMM was prepared from rabbit muscle myosin by the method of Lowey and Cohen (1962). The preparation was examined by alkaline disc gel electrophoresis at pH 8.7 in a 0.05M Tris/glycine buffer on 5 per cent acrylamide gel. The pattern of bands obtained agreed closely with that obtained from a standard preparation of HMM supplied by Worthington Biochemical Corporation. The Worthington HMM was also isolated according to the method of Lowey and Cohen (1962). Myosin was prepared fresh by a combination of methods of Mommaerts and Parrish (1951) and of Perry (1955). Tissue pieces were prepared for decoration with HMM by the method of Spooner *et al.*, 1973. Small pieces of tissue were excised (1-2mm cubes) and placed at 4° in standard salt solution (100 mM KCl/5mM MgCl₂/6mM sodium phosphate buffer pH 7.0), containing 50% glycerol. After 4-12 hours, the concentration of glycerol was lowered to 25 per cent and after a further 4-12 hours it was lowered again to 5 per cent. After another 4-12 hour period, the glycerol solution was replaced by HMM solution (6-8 mg protein/ml) in 15 mM KCl solution containing 10 mM phosphate, pH 7.0. After 24 hour exposure to HMM, the tissue pieces were fixed in 2.5 per cent glutaraldehyde and processed for electron microscopy by the method of Wiggers, *et al.*, (1973). MBs isolated from the livers of two patients with alcoholic hepatitis were also exposed to HMM and processed for electron microscopy. The parotid was used as a positive control. Negative controls consisted of tissue exposed to standard salt solution or standard salt solution containing bovine serum albumin instead of HMM. Following the observations of Schroeder (1973) that ATP and pyrophosphate block HMM binding to actin filaments, the effect of these components on the HMM binding of rat liver filaments was tested. ATP (10 mM) was included with HMM during the filament decoration step. Pyrophosphate (2mM) was also added at the HMM binding step. Cytochalasin B (Calbiochem) was tested in a similar manner using 25 ug/ml in 0.5% dimethylsulfoxide (DMSO) (Butcher and Perdue, 1973). Control liver in 0.5% DMSO without cytochalasin B present were also studied.

The results were similar in both the liver and the parotid tissue. Two types of filaments were noted near the plasma membranes in glycerinated cells. The first formed a lattice network of fine interconnecting filaments near the plasma membrane (Figs. 14-15). These filaments are termed microfilaments (MFs). They appeared to insert into the plasma membrane just as Pollard and Korn (1973) noted in plasma membrane fractions of amoeba. They also appeared to interconnect with various organelles (Comly, 1973). These filaments averaged 5.2 nm. The filaments ran a crooked course and were not tubular. The second type of filament averaged 6 nm thick and was rigid, straight and tubular (Figs. 14 and 15). These filaments are termed intermediate filaments (IFs). These filaments were located at a position more distant from the plasma membrane than the MFs. The IFs appeared to branch occasionally and resembled the type II Mallory body filaments. Similar branching tubular filaments have been described in fibroblasts (Goldman and Follett, 1969). These filaments may accumulate in a sharply demarcated perinuclear zone (Goldman and Follett, 1970) in a manner which resembles an MB. Occasionally, they appeared to insert into mitochondrial membranes, a feature illustrated by Carr (1972) in lymph node macrophages and by De Petris, *et al.* (1962) in macrophages and granulocytes. The lattice network or reticular array type of filament bound HMM in both parotid (Fig. 17) and liver (Fig. 18). Binding of HMM to the MFs has also been reported in other tissues (Pollard *et al.*, 1970; and Spooner, *et al.*, 1973). The IFs did not bind HMM in either liver or parotid (Fig. 17 and 19). Lack of binding of HMM to IFs in other tissues has been described by others (Spooner *et al.*, 1973; Ishikawa *et al.*, 1969; Goldman and Knipe, 1972; Luduena and Wessells, 1973; and Pollard, 1973).

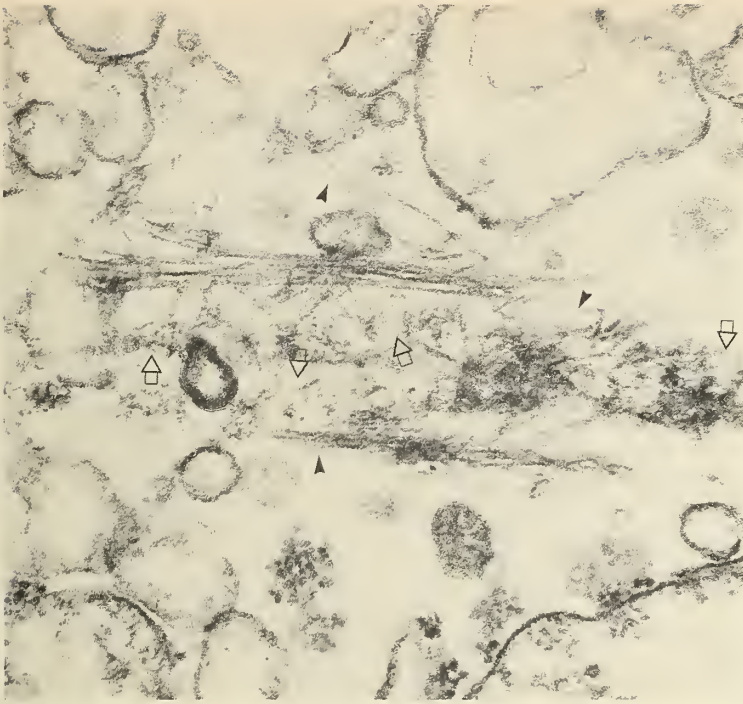


Figure 14. Rat liver which has been extracted by glycerination. Running through the centre from left to right are the plasma membranes of opposing cells. The membranes are cut tangentially so that a distinct membrane is not seen. The microfilament lattice work (5.2 nm in diameter filaments) is seen on either side of the membrane (open arrows). Intermediate filaments (6nm in diameter) are seen flanking the microfilaments (solid arrow heads). EM, 115,500X.

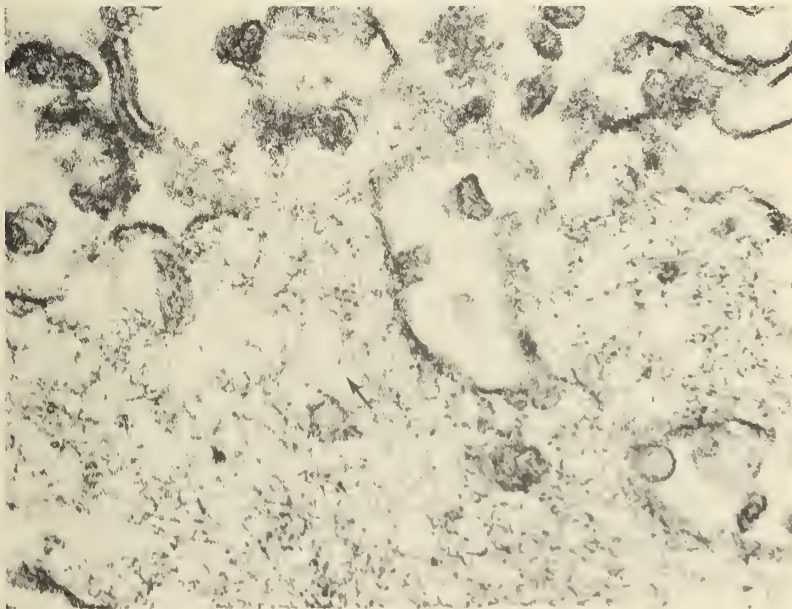


Figure 15. The microfilament lattice work (solid arrow) seen in a rat liver treated with glycerination and incubated with heavy meromyosin (HMM) and ATP. No "decoration" by HMM is seen, EM, 115,500X.

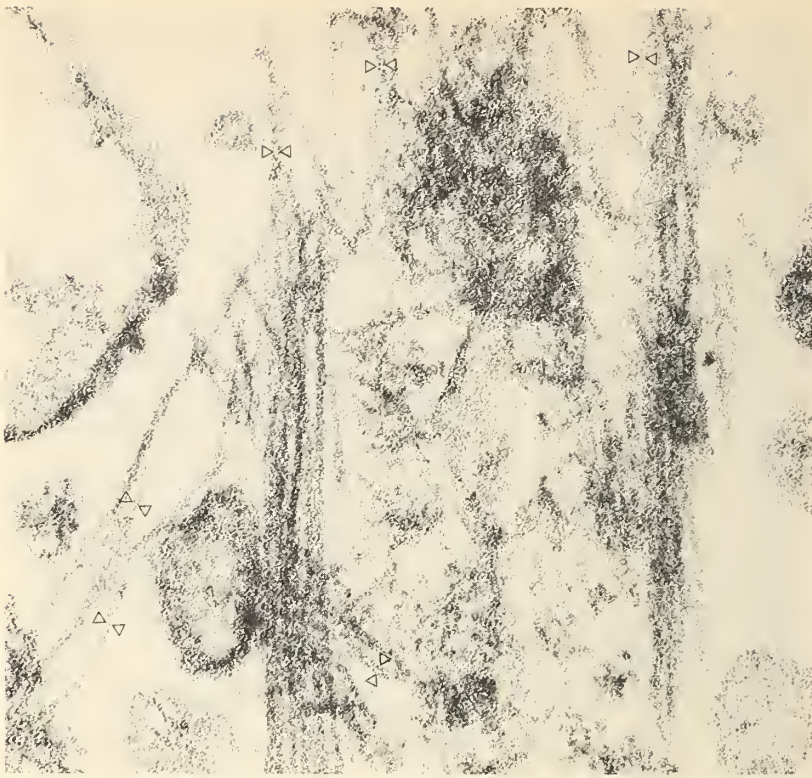


Figure 16. *Higher magnification of fig. 14. Note the tubularity of the intermediate filaments (between open triangles). EM 231,000X.*

However, in every report, the non-HMM-binding IFs measured 10nm thick. ATP blocked HMM binding in the rat liver (Fig. 15). This was expected from the work of Pollard, *et al.* (1971); Goldman, (1973); Luduena and Wessells (1973) and Pollard (1973). Pyrophosphate blockade of HMM binding was only partial. A few decorated filaments were encountered after pyrophosphate, which blocks binding of myosin to actin, but the number of decorated filaments was markedly reduced in comparison to the liver exposed to HMM in the absence of pyrophosphate. Partial inhibition of HMM binding by pyrophosphate was also noted in platelets by Behnke, *et al.* (1971). Cytochalasin B did not block HMM binding. The isolated MB filaments did not bind HMM. Glycerinated liver exposed to bovine serum albumin did not show decoration of filaments.

Thus, it was concluded that normal rat liver cells like cells from other tissues have actin-like filaments beneath the plasma membrane. Normal rat liver cells have a second filament (IF) which morphologically resembles the type II MB filament. Like MB filaments, this second filament did not bind HMM. This is suggestive evidence that MB filaments occur in normal liver cells and these filaments are not F-actin.

A second experiment was done to investigate whether actin-like protein could be extracted from rat liver and be identified by polyacrylamide gel electrophoresis. This was done in order to determine if actin was present in normal liver and in isolated MB preparations. Fractions derived from acetone powder of rat liver were prepared as outlined in Fig. 20. The acetone powder was extracted under depolymerising conditions (F-actin- \rightarrow G-actin). This was achieved in a 1mM solution of ATP containing 0.4 mM CaCl_2 and 1 mM mercaptoethanol, pH 7.5. After gauze filtration and centrifugation at

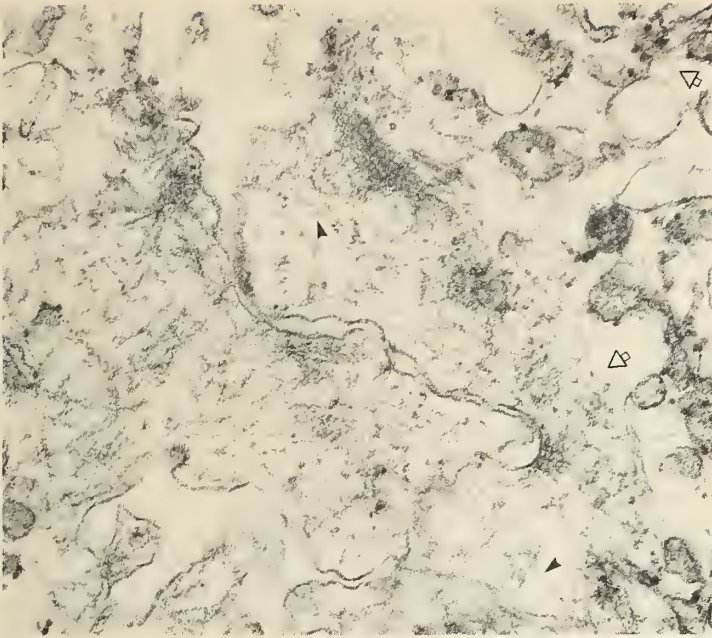


Figure 17. Rat parotid after glycerination and HMM binding to microfilaments (solid arrow heads). Microfilaments now measure 14 nm in diameter due to attached HMM. Flanking the microfilaments are intermediate filaments (open arrows). The microfilaments appear to insert on the plasma membranes while the intermediate filaments extend away from the plasma membrane into the interior of the cell. EM, 69,000X.

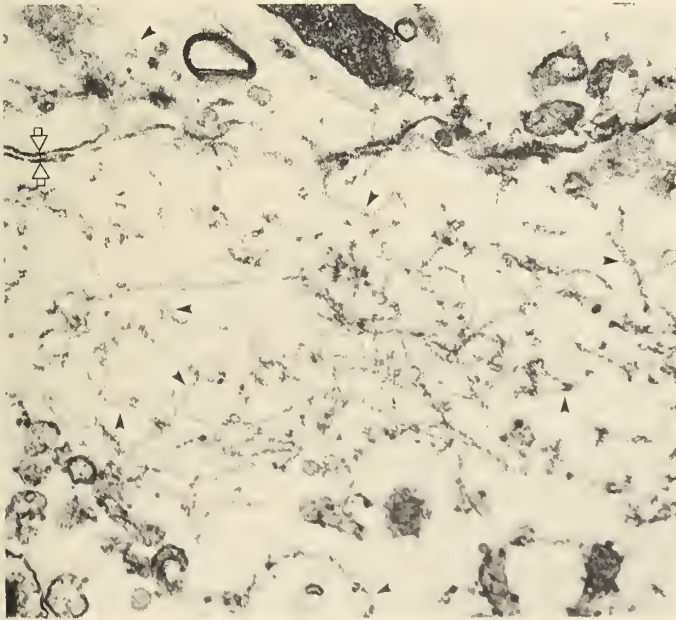


Figure 18. Rat liver after glycerination and incubation with HMM. Note the microfilaments are decorated with HMM (solid arrow heads). The plasma membranes (open arrows) of two opposing cells are broken due to glycerination in order to allow the large molecule of HMM to enter the cytosol during incubation. EM, 75,000X.

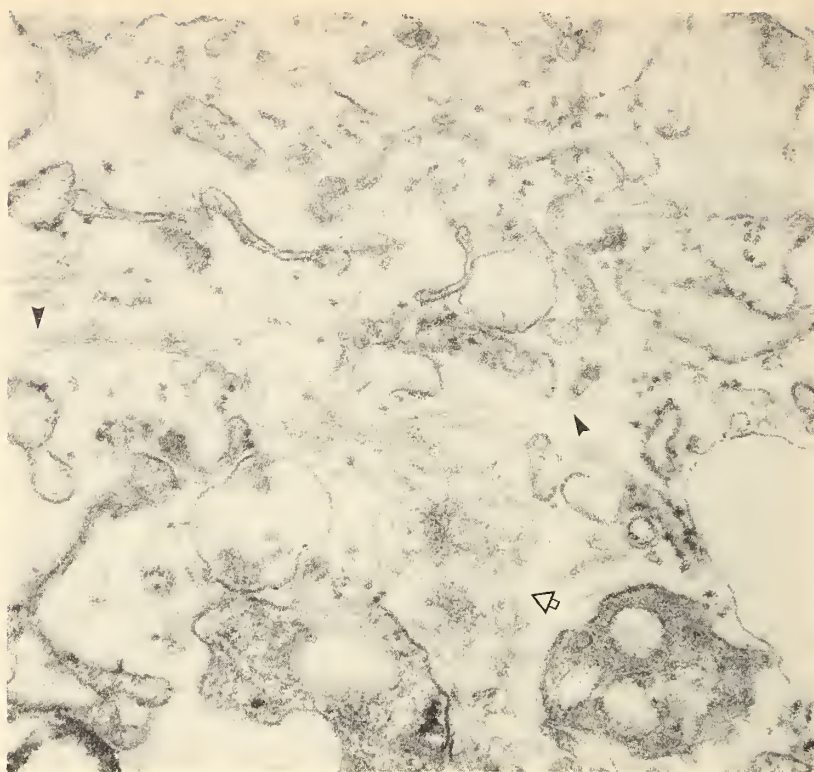


Figure 19. *Rat liver after glycerination and HMM. Note the cluster of decorated microfilaments (open arrow) and undecorated intermediate filaments. EM, 70,500 X.*

13,300 xg for 50 min, fractions 1 and 5 were separated (Fig. 20). High and low molecular weight material and material absorbed to Biogel A 1.5 m were separated by gel filtration under depolymerising conditions (low salt concentration). Material absorbed to the Biogel was eluted with 100 mM KCl solution (high salt concentration) containing 1 mM MgCl_2 and 2 mM Tris, pH 8.0 (fraction 4). The low molecular material was dialyzed to polymerising conditions with the high salt solution used for elution described above. The resulting preparation was centrifuged at 13,300 xg for 20 min. The supernatant was separated into a high and low molecular weight fraction by gel filtration under polymerising conditions. The resulting fractions were examined for the presence of actin-like protein by SDS gel electrophoresis (Weber and Osborn, 1969). Electrophoresis was performed in the presence of 0.1% sodium dodecyl sulphate (SDS) and 0.1 M sodium phosphate buffer (pH 7.0) after denaturation of the sample in the above buffer which contained 4M urea, 1% SDS and 1% mercaptoethanol. The fractions were electrophoresed side by side on an ORTEC slab gel (7.5%) with actin standards (Fig. 21). In this way, it was hoped to observe a protein band in one or more fractions that had the same electrophoretic mobility (and hence molecular weight) as actin. The actin standards were obtained from Worthington Biochemical Corporation or were prepared fresh in our laboratory. In both cases, rabbit muscle was the starting material and the method of isolation used was that of Spudich and Watt (1971). Our actin preparation contained one major band and several minor bands and was not as pure as the actin from Worthington which had only traces of other components (Fig. 21). Of the fractions examined from rat liver, fraction 4 contained an actin-like protein with the same mobility as the standards.

FLOW DIAGRAM FOR EXTRACTIONS OF ACTIN FROM RAT LIVER

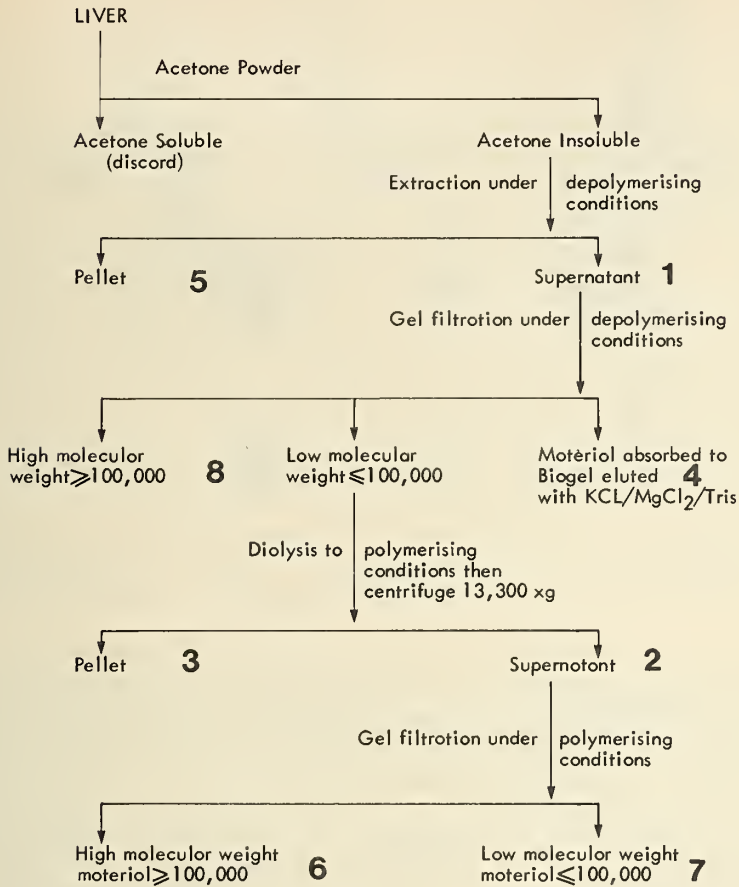


Figure 20. Method used to isolate actin from rat liver. Fractions 1-8 were examined for the presence of actin by SDS gel electrophoresis (see Fig. 21).

This band was also seen in fraction 1 which would have to contain the proteins in fraction 4. These results, although preliminary, indicate that the adult rat liver contains an actin-like protein which according to the HMM binding studies is located in liver cells.

In order to determine whether the isolated MB fraction also contained an actin-like protein SDS gel electrophoresis was performed. The isolated MB fractions obtained from the autopsy of two patients with alcoholic hepatitis were studied. Three control liver fractions were studied in the same way. The MB filaments were dispersed with a solution known to successfully solubilize neurofilaments of squid axons. The solvent system employed was 2M guanidine HCl, 0.05 M B-mercaptoethanol in 0.05M sodium phosphate, pH 7.5 (Humeus and Davison, 1970). The residue not soluble in guanidine was examined for residual MB filaments by light and electron microscopy. No MB filaments were found in the residue which consisted mostly of nuclear material with a few basement membranes and bundles of collagen.¹ The loss of MBs from the residue suggested

¹In more recent studies we have been unable to remove all the MBs with guanidine, i.e. MBs were found in the post-guanidine residue. Yokoo (personal communications) has had similar variable results, i.e. some batches of MBs dissolved in guanidine and other batches did not.

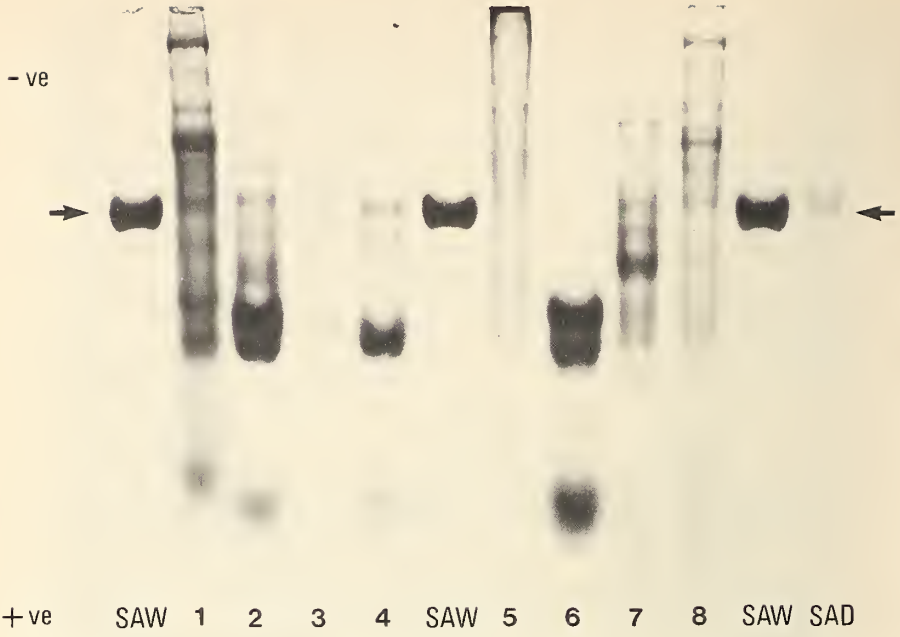


Figure 21. SDS gel electrophoresis of rat liver fractions 1-8 (see fig. 20). The electrophoresis was done on a gel slab (Ortec) The standard actin sample (SAW) was prepared by Worthington from rabbit skeletal muscle. A standard actin sample was also prepared in our laboratory (SAD). Fractions 1, 4 and 7 have a band (solid arrows) which have a mobility similar to actin.

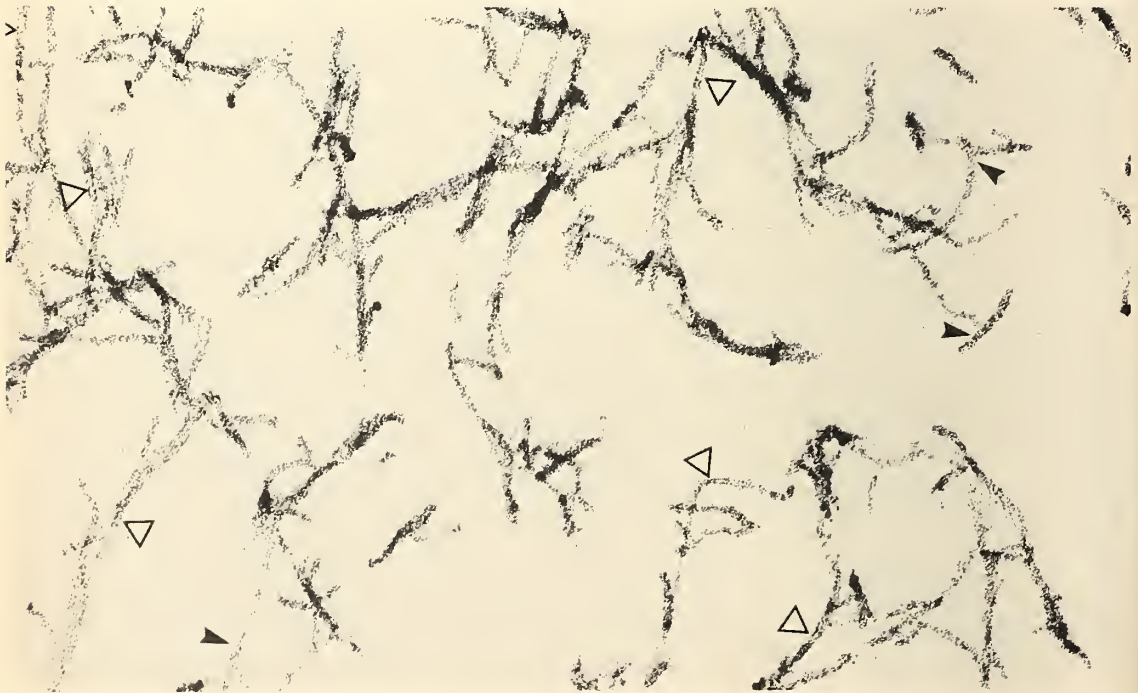


Figure 22. Post dialysis filaments derived from the MB fraction. Branching (solid arrows) and tubularity (open triangles) can be seen. EM, 166,750X.

that the MB filaments were selectively dispersed by the solvent. The solubilized MB protein was dialyzed against water, and was concentrated as a precipitate by centrifugation. Electron microscopy of this precipitate revealed branching filaments (Fig. 22) which varied considerably in width (5.7 to 7.7 nm). The same filaments were found when guanidine extracts of isolated fractions from 3 control livers were dialyzed against water suggesting that MB protein existed in normal liver. However, the isolated fraction from control livers before extraction did not contain MB filaments by electron microscopy examination. The dialyzed MB protein extracts were denatured in a buffer solution of 0.1 M sodium phosphate buffer, pH 7.0, which contained 4 M urea, and 1% SDS and 1% mercaptoethanol. Gel electrophoresis was performed in the presence of 0.1% SDS as described above for actin using 7.5% polyacrylamide gel.

Three Coomassie blue positive bands were observed in the MB extract and two were seen in the controls (Fig. 23). Both the extracts showed a high molecular weight (MW) band estimated to be 120,000 to 123,000 MW using standards of known M.W. This band was well delineated and in relatively high concentration. The low molecular weight bands were less well defined. The band which was found only in the MB extract showed an intermediate electrophoretic mobility. When MB extracts were run together with the actin standard on the ortec slab in SDS it was established that a protein with the mobility similar to actin was not present in the MB extract. Since filaments form during dialysis of both the MB and control extracts, the middle band seen only in the MB extract is not essential for the formation of filaments.



Figure 23. SDS gel electrophoresis of the 2M guanidine HCl extract of an isolated MB fraction (right) and two control livers (left).

It is interesting to note that the high molecular weight bands have molecular weights near that of the native dimer of tubulin (110,000 and 130,000 daltons — Shelanski and Taylor, 1968). The IFs of smooth muscle also contain two high molecular weight proteins (90,000 and 105,000 daltons, Rice and Brady, 1972). The MW of the subunit of 8-9 nm neurofilaments (filarin) is approximately 80,000. The subunits from these various sized fibers probably differ in chemical composition (Shelanski, 1973). MB filaments resemble closest the neurofilament in that they have similar diameters, tubularity, insolubility at

different ionic and H^+ ion concentrations and solubility in 2M guanidine HCl. An MB-like collection or proliferation of neurofilaments in nerve cells can be induced by aluminum (Shelanski and Taylor, 1970; Shelanski and Feit, 1972; Wisniewski and Terry, 1970 and Wisniewski, Terry and Hirano, 1970), which may be etiologically related to Alzheimer's disease (Crapper, Krishnan and Dalton, 1973).

It seems likely that one of the MB derived proteins separated by MB isolation-guanidine extraction-dialysis-SDS gel electrophoresis is the subunit of the MB filament, especially since it polymerized *in vitro* to form branching, tubular filaments which resembled MB filaments. However, other interpretations are possible because a filament-forming protein was also extracted from isolated fractions of normal livers. It is not clear which of the high or the low M.W. protein bands is the MB filament subunit. Therefore, a method must be found to positively identify the MB filament subunit as the protein extracted by guanidine. This could be accomplished by inducing a specific antibody by sensitizing rabbits to the MB proteins. The antibody could then be tagged by conjugation with horseradish peroxidase and the labelled antibody could be exposed to tissue slices of livers containing MBs. If the labelled antibody localizes to bind MB filaments this would identify MB protein subunits in the MBs. Similarly, if the antibody binds IFs in normal liver cells and other cell types this would prove that MB filaments are indeed composed of normal IFs.

THEORETICAL CONSIDERATIONS

Pathogenesis of Mallory Bodies

If the assumption is made that MB filaments are derived from a normal protein constituent of the liver cell cytosol then it should be possible to characterize MB filaments by comparing their chemical and morphologic features with those of normally occurring filaments. The evidence reviewed above indicated that MB filaments resemble best the intermediate filament (IF). This filament does not bind HMM, appears tubular, has a branching architecture and has a diameter close to that of the MB filaments. The IFs measured 6nm in the studies reported here and measured 8-10 nm in studies reported by others (Taylor, 1966; Goldman and Follett, 1969; Ishikawa, *et al.*, 1969; Goldman and Knipe, 1972; Pollard, 1973 and Spooner, *et al.*, 1973).

Using the same logic employed above, we might be able to predict why MB filaments accumulate in alcoholic hepatitis by reviewing the factors which are known to influence the accumulation of IFs in cells. First, consider the observation that IFs are not usually visible in rat liver cells which have been prepared for electron microscopic examination by routine methods. Following glycerination, however, they are easily found at the cell periphery separated from the plasma membrane by a thin zone of MFs. IFs are also seen in small numbers between organelles. Numerous authors have observed that glycerination (glycerol extraction in a low ionic strength solution at 22°) or exposure of cells to a hypotonic solution (osmotic shock) makes either the MFs or IFs become more conspicuous or appears to make the filaments increase in number (Taylor, 1966; Zucker-Franklin, 1969; Goldman and Follett, 1970; Pollard, *et al.*, 1970; Zucker-Franklin, 1970; and Behnke, *et al.*, 1971). This increase in cytoplasmic filaments could result from extraction of background material which otherwise masks filaments, or it may be due to the assembly of filaments from monomers which ordinarily are not polymerized (Behnke, *et al.*, 1971).

Although microtubules, MFs and thick filaments (myosin) appear or disappear as the microenvironment influences their assembly and disassembly, the IFs appear to change their intracellular distribution but not their amount (Goldman and Knipe, 1972; and Fay and Cooke, 1973). Goldman and Knipe (1972) showed that living BHK 21 cells develop a perinuclear spherical collection of IFs (10 nm in diameter) 15-30 minutes after contact with substrate. At this time the organelles were mostly excluded from the IF area and MFs and microtubules were few in number. After these cells were allowed to spread, the IFs were distributed throughout the cytoplasm and large numbers of MFs and microtubules were formed. The redistribution of IFs and the assembly of microtubules and MFs was not inhibited by the protein synthesis inhibitor cycloheximide indicating that *de novo* protein synthesis was not involved.

The perinuclear spherical collection of IFs failed to disperse in spreading cells when microtubule formation was blocked by colchicine (Goldman and Knipe, 1972). By analogy, this suggests that the accumulation of MB filaments which also accumulate in a perinuclear position could result from a failure of microtubules to disperse the MB filaments.

Reversible assembly and disassembly of MFs and thick filaments appear to be controlled by changes in calcium concentration, whereas IFs (10 nm thick) appear unaffected (Fay and Cooke, 1973). There is also evidence that polymerization of tubulin to form microtubules is controlled by calcium levels (Weisenberg, 1972). Little is known about the factors which govern the assembly and disassembly of IFs. They differ from the other filaments in that they are insoluble even in solutions of high ionic strength (IFs of smooth muscle, Cooke and Chase, 1971) and have low solubility in SDS (IFs of squid axons, Huneus and Davison, 1970).

The diameter of intracellular filaments cannot be used as a reliable criterion to predict a specific protein subunit composition of the filament (Behnke, *et al.*, 1971). The thickness of myosinoid filaments can vary from 4 to 180 nm depending on the pH, ionic strength and concentration of calcium and magnesium of the medium (Behnke, *et al.*, 1971). Interconversion of filaments of various sizes can be affected by a change in pH at a given ionic strength (Kaminer and Bell, 1966; and Morgan, Fife and Wolpert, 1967). Further, filaments of any given morphologic class vary considerably in their diameters indicating a single monomer can assemble into filaments which vary in the number of subunits per turn of spheres packed in tubular arrangement (Erickson, 1973). For instance, actin monomers polymerize in physiological salt solution to form 6 nm wide filaments yet actin filaments in amoeba measure 8 nm wide (Pollard and Korn, 1972). Therefore, the classification of cytoplasmic filaments as 6, 10, and 25 nm filaments (Goldman and Knipe, 1972) gives no real insight into the chemical composition of the filaments. The identification of the normal equivalent of MB filaments can be better based on solubility and specific binding characteristics and chemical composition rather than similarity in ultrastructure.

In summary, MB filaments are more likely to represent pathological accumulations of IFs rather than MFs or thick filaments for the following reasons: (1) they have similar morphologic characteristics such as thickness, branching and tubularity; (2) they occur in the same location within the cell; (3) they do not bind HMM; (4) they are insoluble in solutions of varying ionic strength and pH; (5) they are soluble in 2M guanidine HCl; (6) they appear to contain high molecular weight proteins.

The pathogenesis of MBs remains a mystery. Since they are found in cells which appear otherwise normal, it is likely that overhydration of hepatocytes is not the cause. Since they occur in extrahepatic cells such as pneumocytes it is likely that they represent

a fundamental but nonspecific reaction to injury. If the accumulations of neurofilaments in nerve cells induced by aluminum toxicity are comparable to MB formations in liver cells then an experimental model is available for the study of MB formation.

As a working hypothesis the following pathogenetic scheme is offered: 1. MBs are derived from IFs. 2. IF protein monomers, which are normally synthesized by the granular endoplasmic reticulum, polymerize at the site of synthesis (Figs. 9-10). 3. Normally, microtubules disperse the IFs throughout the cytosol. This process fails to occur in the injured liver cell of alcoholic hepatitis or cirrhosis. IFs continue to grow and expand as a mass displacing adjacent organelles (Fig. 4). This hypothesis could conceivably be tested by interfering with microtubule assembly using colchicine. Theoretically, colchicine should block the dispersal of IFs by microtubules through its depolymerising effect on microtubules (Goldman and Knipe, 1972). This could be the mechanism for IF proliferation observed in cells (Shelanski and Feit, 1972). However, Stein and Stein (1973) did not note an accumulation of IFs in livers of rats treated with colchicine. Other endogenous changes in the microenvironment such as levels of cAMP, cGMP, calcium, magnesium, GTP or ATP may modulate the assembly and function of microtubules (Weisenberg, 1972; Kram and Tomkins, 1973; Margulis, 1973; Shelanski, 1973; and Zurier, Hoffstein and Weissmann, 1973) and therefore could conceivably influence the intracytoplasmic distribution of IFs.

An alternative hypothesis is that the MBs form because the IFs are assembled in an abnormal way which makes the IFs non-dispersable. It has been shown that the MB holds together as a single unit of branching, cross-connecting filaments (Figs. 12 and 13). This accounts for the fact that they can be isolated intact in a subcellular fraction whereas normal IFs cannot be isolated in this way. If the MB filaments are so tightly connected to each other so that they cannot be mechanically dispersed by homogenization, it is reasonable to expect that the normal *in vivo* mechanism of dispersal would fail to achieve the distribution of the MB filaments throughout the cytoplasm. This explanation fails to answer the question why the MB filaments assemble as a branching unit.

Functional Consequences of MB Formation

If the MB develops in liver cells that are otherwise normal in appearance, why do these cells go on to develop maldistribution of organelles (Figs. 4 and 10), accumulate fat (Figs. 9 and 10) and ultimately undergo cell death (Fig. 3)? It seems likely that MB formation leads to, or is associated with, loss of cell function. For instance, the accumulation of fat in these cells could result from the failure of the exocytosis apparatus to secrete lipoproteins. Stein and Stein (1973) showed that colchicine inhibited the release of lipoprotein from the rat liver *in vivo*, presumably by disrupting the microtubular dependent exocytosis of secretory vesicles. This study indicated that only the final step in intracellular transport and secretion was affected by microtubular depolymerisation. Secretory vacuoles filled with very low density lipoprotein derived from the Golgi apparatus accumulated in the liver cells and the release of labelled fatty acid into the blood was diminished. Colchicine has a similar inhibitory effect on a variety of secretory and exocytosis processes (Gillespie and Lichenstein, 1972; Plaut, Lichenstein and Henney, 1973; and Taylor, Mamelak, Reaven, and Maffly, 1973). Cyclic nucleotides also appear to control the release of secretory products by exocytosis (Ignano and Columbo, 1973; Taylor *et al.*, 1973; and Zurier *et al.*, 1973). This indicates that factors which influence the secondary messenger system, or the plasma membrane receptor sites which trigger it, could also be involved in failure of liver cells to release lipids.

Wagner and Rosenberg (1973) also showed that the microtubule inhibitor colcemid inhibited the movement of formed vacuoles to the cytocenter of Chang liver cells with concomitant disappearance of microtubules. Inhibition of saltatory movements of formed endocytotic vacuoles within the cytoplasm by microtubule inhibition has also been demonstrated in other cell types beside the liver (Freed and Lebowitz, 1970; and Bhisey and Freed, 1971, a & b). Microtubule inhibitors affected both the movement and the orientation of various cytoplasmic formed elements. Thus, the transhepatic transport by way of the cytocavitary system could be inhibited by a process which interferes with microtubular function. The *in vivo* existence of this cytocavitary system which transports material from the space of Disse to the Golgi apparatus and then transports it to be secreted into the bile canaliculus or digested in digestive vacuoles has been established by Matter, Orci and Rouiller (1969).

One of the ultrastructural changes observed after colcemid disassembly of microtubules in Chang liver cells was a striking disorientation in filamentous mitochondria. These mitochondria appeared to become intertwined with one another and vacuoles became trapped amongst these snarled organelles. If liver cells containing MBs were similarly affected, that is, the cell cytocavitary system was without microtubular guidance and motive force, we might expect to see in such cells evidence of organelle disarray including distortion and maldistribution of mitochondria. Such a pattern of disorder is often seen in cells containing MBs (Figs. 4 and 10). This disarray of organelles contrasts sharply with normal human liver cells (Fig. 24). Often the long snake-like column of MBs compartmentalized the cytoplasm of the involved cells (Fig. 10) suggesting that the MB may have formed a physical barrier to the movement of organelles.

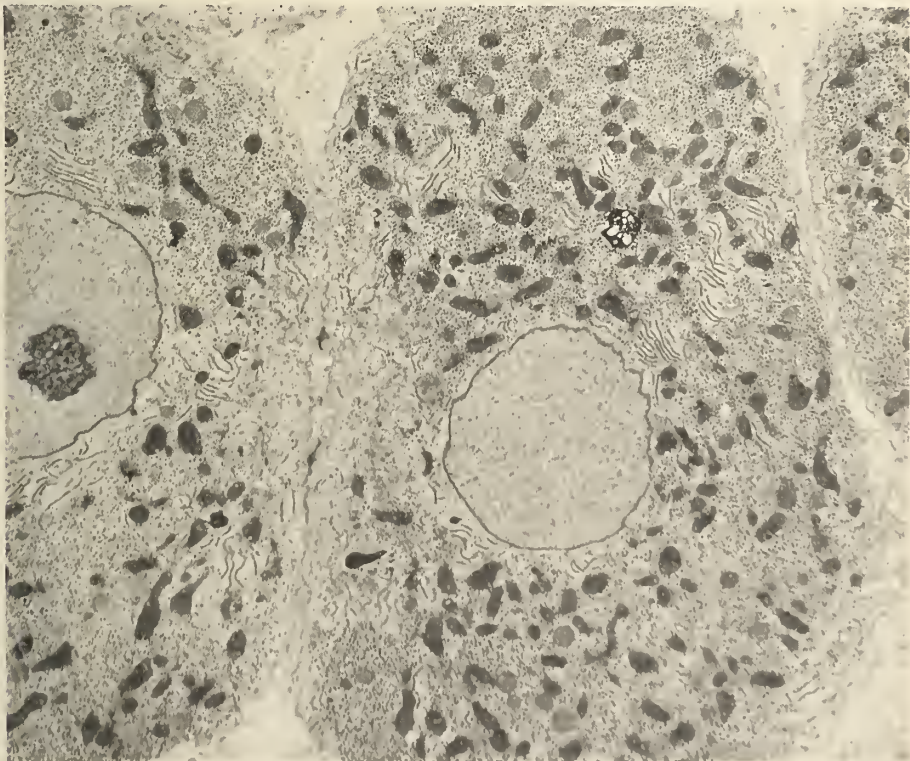


Figure 24. Liver biopsy showing normal liver cells. Note the rather even distribution of organelles and glycogen throughout the cytoplasm except around the bile canaliculus. EM, 5950X.

If the degenerative changes observed in liver cells containing MBs are due to a disorder of microtubules, how is this related to MB formation? There is not enough evidence available to answer this question. It may be that the MB filament accumulation sequesters the monomers involved in microtubular function. A number of investigators have postulated that IFs and microtubules share the same subunits or can be interconverted (Peters and Vaughn, 1967; Zucker-Franklin, 1969; and Bhisey and Freed, 1971, a and b). This hypothesis is based on the fact that IFs increase in number or become more apparent when microtubules are depolymerized by colchicine or are otherwise made to disappear (Zucker-Franklin, 1969; Bhisey and Freed, 1971, a and b; and Wuerker and Kirkpatrick, 1972). However, subunits of at least one type of IFs (filarin) differ chemically from those of microtubules (tubulin) except for those filaments induced by spindle inhibitors (Shelanski and Feit, 1972; Shelanski, 1973). We noted that protein extracted from MB filaments had a molecular weight similar to the dimer of tubulin (120,000). However, the dimer of tubulin should have dispersed into two monomers under the denaturation conditions that we used to study the MB extract (SDS, urea and mercaptoethanol) unless the tubulin dimer was altered in such a way that it resisted disaggregation. Resolution of this problem will require better characterization of the MB protein.

Lastly, it is possible that the IFs which seem to constitute the MB filaments may have a function of their own which is lost when their subunits are consumed by MB filament formation. There is evidence that IFs and microtubules are closely associated and therefore have complementary or cooperative functions. Goldman and Knipe (1972) postulated that microtubules and IFs form a complex involved in the intracellular transport of organelles along the long axis of fibroblasts. A similar association has been implicated in the movement of organelles in axons (Wuerker and Palay, 1969; and Weiss and Mayr, 1971).

SUMMARY

Since the first description by Mallory in 1911, the nature, pathogenesis, natural history and functional implications of Mallory bodies (MBs) have eluded the grasp of investigators. In this paper, what is known about Mallory bodies was reviewed, new information was reported and a working hypothesis was offered. Drawing from this information the following summary is constructed.

1. Mallory bodies are not unique to liver cells or to alcoholic hepatitis.
2. They are composed of branching filaments which are of two types.
3. The type II Mallory body resembles the intermediate filaments (IFs) which are found in most cell types investigated including unicellular organisms.
4. The type II filaments share the ultrastructural and solubility characteristics of IFs.
5. Proteins extracted from MB filaments can be isolated by gel electrophoresis from liver fractions of isolated MBs and controls. These proteins probably form branching MB-like filaments indicating MBs can probably be reconstituted *in vitro* from proteins present in normal liver.
6. Histochemical studies indicate that MBs are composed of insoluble basic proteins but further characterization of MBs by this approach is limited, due to the fact that MBs entrap a large variety of extrinsic material including organelles.
7. Although normal liver cells appear to contain actin-like microfilaments, MBs do not bind HMM and do not contain actin by SDS gel electrophoresis.

8. MBs are seen in association with granular endoplasmic reticulum (GER) at a time when the liver cell is otherwise normal in appearance. The filaments probably form from subunit polymerisation in niduses which result from changes in the microenvironment.

9. As the mass of MB filaments grows in size, the association with GER becomes less obvious and surrounding organelles are displaced. Ultimately the liver cell cytoplasm becomes compartmentalized by the MBs and the cytosol becomes filled with small fat vacuoles or amorphous material devoid of glycogen. At this time, the mitochondria become maldistributed and distorted. PMNs and monocytes appear to invade the affected liver cells forming the MB-PMN complex. The latter is one of the hallmarks of alcoholic hepatitis. This MB-PMN complex appears to be one of the factors which contributes to hepatic fibrosis and cirrhosis at least in the alcoholic patient.

10. The fate of the cell containing MBs appears to be cell death but there is some evidence that dispersion of MB filaments may occur.

11. From what is known of the function of IFs and microtubules it is postulated that MB formation interferes with the function of liver cells by reducing their transport and secretory functions.

ACKNOWLEDGEMENT

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Hepatic Fibrogenesis in Alcoholism

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INTRODUCTION

Chronic alcoholism is a principal cause of hepatic cirrhosis (Klatskin, 1961). The development of cirrhosis in chronic alcoholics appears to correlate somewhat with the length of time and amount of abuse of alcoholic beverages (Leibach, 1967). However, only a small percentage of chronic alcoholics (8 to 20%) eventually develop cirrhosis (Klatskin, 1961; Leevy and tenHoven, 1967), suggesting that other factors, either dietary, toxic, or genetic, are of importance in pathogenesis.

Extensive investigations in both animals and humans have clarified the pathophysiology of fatty infiltration of the liver in chronic alcoholism (Lieber, Jones and DeCarli, 1967; Porta, Koch and Hartroft, 1970; Rubin and Lieber, 1968). Fatty liver is basically a reversible condition and there is no evidence that fat predisposes to cirrhosis (Popper, 1961; Shorter and Baggenstoss, 1959). On the other hand, alcoholic hepatitis has been shown to precede the development of cirrhosis (Galambos, 1972; Helman, Tenko, Nye and Fallon, 1971).

The deposition of excess connective tissue in the liver is a hallmark in the development of chronic liver disease. The collagen content of cirrhotic livers at autopsy has been demonstrated to be five to ten times greater than that found in normal livers (Hutterer, Rubin, Singer and Popper, 1959; Kent, Fels, Dubin and Popper, 1959; Morrione, 1947). The study of the pathogenesis of liver cirrhosis in chronic alcoholism has been hindered by the lack, up to the present, of a suitable animal model in which ethanol is associated with the development of hepatic fibrosis; however, induction of experimental hepatic cirrhosis by feeding of deficient diets or toxic substances other than ethanol have helped clarify the pathogenesis of the deposition of fibrous tissue in the liver.

Studies by several investigators suggest that increased collagen deposition, and not collapse of hepatic reticulum, initiates hepatic fibrosis (Popper and Udenfriend, 1970). The increased collagen deposition could result from either increased collagen synthesis, decreased degradation, or both. The biosynthesis of collagen involves the formation of a polypeptide intermediate, rich in the amino acids proline and lysine, which has been termed "procollagen". These amino acids are hydroxylated to hydroxyproline and hydroxylysine, respectively, while the procollagen is still attached to the ribosomes prior to the formation of the triple helix structure of collagen (Grant and Prockop, 1972). The hydroxylation reactions are catalyzed by two separate enzymes present in the soluble fraction of cells synthesizing collagen (Miller, 1971). Most thoroughly studied up to the present time has been the hydroxylation of proline by procollagen proline hydroxylase. Increases in the activity of procollagen proline hydroxylase have been shown to precede collagen deposition in the skin, during experimental granuloma formation and wound healing, suggesting that proline hydroxylation is a rate limiting step in collagen synthesis (Mussini, Hutton and Udenfriend, 1967). Increases in procollagen proline hydroxylase have also been found in the skin of some patients with collagen disorders (Uitto, Hannuksela and Rasmussen, 1970), in the lungs in experimental pulmonary fibrosis (Halme, Uitto, Kahanpää, Harhunen and Lindy, 1970), and in the pregnant uterus of the mouse (Halme and Jäskeläinen, 1970).

Studies of experimental carbon tetrachloride induced hepatic fibrosis in rats have demonstrated increases in procollagen proline hydroxylase activity (Takeuchi, Kivirikko and Prockop, 1967; Takeuchi and Prockop, 1969), in the incorporation of ^{14}C proline by liver slices (Huberman, Recio and Rojkind, 1969; Rojkind and Diaz de Leon, 1970), and in the hepatic content of hydroxyproline (Huberman, Recio and Rojkind, 1969; Rojkind and Diaz de Leon, 1970; Takeuchi, Kivirikko and Prockop, 1967; Takeuchi and Prockop, 1969). The increases in procollagen proline hydroxylase and in the hepatic content of hydroxyproline were found as early as two weeks after the bi-weekly administration of carbon tetrachloride (Takeuchi and Prockop, 1969). Increases in the activity of the enzyme and in the hepatic content of hydroxyproline have also been observed in rats following induction of hepatic fibrosis by a choline deficient diet (Takeuchi and Prockop, 1969). More recently similar increases in procollagen proline hydroxylase, in the incorporation of ^{14}C proline into hepatic collagen, and in hydroxyproline content were demonstrated in the livers of rats and baboons after chronic ethanol feeding. However, no fibrosis could be detected by light or electron microscopy in these animals (Feinman and Lieber, 1972). An increased incorporation of ^3H proline into collagen has been shown in the presence of ethanol *in vitro* by liver biopsy samples from patients with alcoholic hepatitis and cirrhosis, but not in those with fatty liver (Chen and Leevy, 1972).

Most body collagen is relatively inert (Neuberger and Slack, 1953); however, following increased collagen deposition secondary to experimental injury of certain tissues such as skin (Jackson, 1957), and liver (Morrione, 1949) the degradation of collagen can become quite rapid. During the early development of hepatic fibrosis induced in rats with carbon tetrachloride there is a parallel increase in the number of parenchymal cells and collagen content estimated from DNA and hydroxyproline determinations respectively (Rubin, Hutterer and Popper, 1963). More advanced fibrosis and cirrhosis is characterized by a more rapid increase of collagen content than the increase in the number of parenchymal cells. While the early development of fibrosis is rapidly reversible, the more advanced cirrhosis is associated with a prolonged half-life of insoluble collagen compatible with a decrease in the rate of collagen catabolism (Hutterer, Eisenstadt and Rubin, 1970).

A similar increase in the half-life of insoluble collagen has also been observed in choline deficiency induced cirrhosis in rats (Takada, Porta and Hartroft, 1967). The determination of the urinary excretion of hydroxyproline provides an estimate of the degradation of body collagen (Prockop and Kivirikko, 1967; Weiss and Klein, 1969), since hydroxyproline liberated during collagen reconstruction is not reutilized but excreted in the urine (Weiss and Klein, 1969). Most of the hydroxyproline excreted in the urine is derived from bone, and increases in its excretion are mostly observed in disorders of bone (Dull and Henneman, 1963). However, increases in urinary hydroxyproline have been observed also in endocrinological disorders (Benoit, Theil and Watten, 1963), in collagen diseases (Emmrich, Häntzschel and Häntzschel, 1967; Ziff, Kibrick, Dresner and Gribetz, 1965), during the induction of liver cirrhosis in rats fed a choline deficient diet (Iturriaga, Peterman, Ugarte and Iriarte, 1967), and in patients with cirrhosis of the liver (Kratzsch, 1969).

The present study deals with determinations of the activity of hepatic procollagen proline hydroxylase and the urinary excretion of hydroxyproline as parameters reflecting collagen turnover in patients with various types of alcoholic liver disease.

PATIENTS AND METHODS

Forty-four chronic alcoholic patients ranging in age from 32 to 74 years (mean, 46.3 years) were studied. The patients were admitted to the medical wards of the Baltimore City Hospital and the Johns Hopkins Hospital because of alcoholism related illness, and required liver biopsies for diagnostic purposes. Twelve were female and thirty-two male. Their history of alcoholism varied from 5 to 25 years, and all had been drinking in excess of 200 grams of ethanol daily for at least 3 days prior to admission. None of the patients had a history of narcotic abuse or recent ingestion of barbiturates or tranquilizers. On admission to the hospital the patients were placed on a regular hospital diet, and were managed on the medical service. Liver biopsies for histological examination and determination of the activity of procollagen proline hydroxylase, followed one to two days later by a 24 hour urine collection for the determination of hydroxyproline excretion were obtained within one week of admission to the hospital.

Seven non-alcoholic patients in whom liver biopsies were obtained for diagnostic purposes served as controls. Their ages ranged from 22 to 49 years (mean, 37.2 years). Two were female and five male. Histological examination of the liver biopsies in these control patients revealed mild portal inflammation in one, non-caseating granuloma in two, and normal liver in the remaining four.

Liver biopsies were performed with a Klatskin modification of the Menghini needle. In most cases two specimens were obtained. Part of the tissue was fixed in 10% formaldehyde for histological examination, and the remainder weighed and homogenized. The samples weighing 10 to 35 mg were homogenized at 0° C in a Potter-Elvehjem apparatus with 1.2 ml of 0.1M Tris-HCl buffer, pH 7.5. Procollagen proline hydroxylase activity was assayed by the method of Hutton, Tappel and Udenfriend (1966), using the modified cofactor concentrations and standardization of the assay introduced by Stein, Keiser, and Sjoerdsma (1970). This method measures tritiated water formed when tritiated proline present in the procollagen substrate is hydroxylated to hydroxyproline. The reaction mixture consisted of: procollagen labelled substrate, 0.5 ml; tissue homogenate, 0.5 ml; α -ketoglutarate, 1mM; ascorbic acid, 5 mM; ferrous ammonium sulfate, 1 mM; all in a volume of 2.5 ml of 0.1 M Tris-HCl buffer, pH 7.5. The reaction mixture was

incubated aerobically for 30 min at 30°C, and then stopped by the addition of 0.2 ml of 50% trichloroacetic acid (TCA). The tritiated water produced was separated by vacuum distillation and counted in Bray's solution in a Packard Tri-carb scintillation counter, model 3375. Quenching was corrected by the use of internal standards. The efficiency of counting was an average of 18 per cent. A blank obtained by the addition of TCA immediately prior to incubation of the reaction mixture (zero time) was run each time. A standard procollagen proline hydroxylase enzyme extract from rat skin was also assayed with each determination to insure uniformity of the assay on different days. The reaction was linear up to 30 minutes with liver tissue between 5 and 30 mg wet weight. Enzyme activities were expressed as d.p.m. per mg of protein or per gram of wet liver weight. The concentration of protein was determined by the method of Lowry *et al.* (1966) with bovine serum albumin used as a standard.

The procollagen labelled substrate was prepared by incubating tibias of 9 day old chick embryos with 3,4 -T- labelled proline (New England Nuclear Corporation, Boston) in the presence of 1 mM α , α -dipyridyl in Krebs-Ringer buffer as described by Hutton, Tappel and Udenfriend (1966). Ten ml aliquots of the substrate were stored at -10° C and thawed immediately prior to use. The substrate solutions contained 1-2 mg of protein per ml with specific activity varying between 75,000 and 200,000 c.p.m. per mg of protein. The standard procollagen proline hydroxylase extract was prepared from fetal rat skin by the method of Hutton, Tappel and Udenfriend (1967).

The content of total hydroxyproline in the twenty-four hour urine collections was determined by the method of Kivirikko, Laitinen and Prockop (1967).

The type of alcoholic liver disease found in the patients was diagnosed according to histological criteria as either fatty infiltration of the liver, alcoholic hepatitis, inactive cirrhosis or non-specific inflammation. All slides were stained with hematoxylin-eosin and Masson's trichrome, and reviewed without knowledge of the clinical findings, first independently by two of us (EM and WCM), and then jointly to resolve any disagreements. Fatty infiltration of the liver was only diagnosed when it was not associated with features of alcoholic hepatitis or significant fibrosis. The percent fatty infiltration of the liver parenchyma was graded as follows: 0, none; trace, 0 - 5%, +1; 5 - 25%, +2; 25 - 50%, +3; 50 - 75%, and +4, more than 75%. The criteria for the diagnosis of alcoholic hepatitis included parenchymal necrosis and inflammation principally with leukocytes. The presence of fatty infiltration, alcoholic hyaline, and fibrosis was noted but not deemed necessary for the diagnosis. The degree of fibrosis was graded according to Lischner, Alexander and Galambos (1971) as follows: +1, peripheral and around the central vein; +2, scattered parenchymal; +3, confluent with the formation of thin septa; and +4, thick fibrous septa with nodule formation. Inactive cirrhosis was diagnosed by the finding of thick fibrous septa and nodular regeneration in the absence of necrosis and inflammation. Non-specific changes not fitting into the classical categories of alcoholic liver disease described above were either periportal mononuclear inflammation or scattered areas of focal parenchymal necrosis. The results are expressed as means \pm 1 SEM. Significance of differences in values were analyzed by the students t test (Bancroft, 1957).

RESULTS

Among the 44 alcoholic patients studied, 10 had fatty infiltration of the liver; 4, mild non-specific changes; 25, alcoholic hepatitis; and 5, inactive cirrhosis. The age range of the patients in the different categories was similar except for the patients with inactive

cirrhosis of the liver, who were older (Table I). Hepatomegaly (liver edge extending between 2 and 15 cm below the right costal margin at the midclavicular line) was a finding in all but 2 of the patients. These 2 patients had non-specific changes in the liver biopsy. Splenomegaly and/or ascites were only present in a few of the patients with alcoholic hepatitis, and each in one patient with inactive cirrhosis. Abnormalities of liver function were more common and marked in the patients with alcoholic hepatitis (Table II).

TABLE I.
CLINICAL FEATURES IN THE FORTY-FOUR ALCOHOLIC PATIENTS

Liver Histology	Patients	Age	Hepatomegaly	Splenomegaly	Ascites
	no.	years	no.	no.	no.
Fatty Infiltration	10	36-56	10	0	0
Non-specific Changes	4	23-58	2	0	0
Alcoholic Hepatitis	25	32-61	25	4	5
Inactive Cirrhosis	5	43-74	5	1	1

TABLE II
LABORATORY DETERMINATIONS OF LIVER FUNCTION IN THE
FORTY-FOUR ALCOHOLIC PATIENTS

Liver Histology	Number of Patients	Serum Bilirubin (mg/100 ml)	SGOT* (I.U./ml)	Serum Albumin (g/100 ml)
Normal Values		no. Abnormal (range) (>1.1)	no. Abnormal (range) (0-19)	no. Abnormal (range) (0-3.5)
Fatty Infiltration	10	4 (1.7-12.2)	5 (37-91)	3 (2.7-3.4)
Non-Specific Changes	4	0	2 (24-56)	1 (2.4)
Alcoholic Hepatitis	25	22 (1.3-15.6)	25 (34-171)	11 (1.7-3.4)
Inactive Cirrhosis	5	3 (1.4-4.1)	3 (31-82)	3 (2.6-3.4)

*SGOT, Serum glutamic oxalocetic transaminase. I.U., International units.

The mean concentrations of protein per gram of liver wet weight in the various groups were not significantly different. They were as follows: normal, 109.0 ± 11.1 mg/g; fatty infiltration of the liver, 114.5 ± 13.8 mg/g; non-specific changes, 124.2 ± 30.2 mg/g; alcoholic hepatitis, 89.1 ± 9.5 mg/g; and inactive cirrhosis, 119.5 ± 15.1 mg/g.

PROTOCOLLAGEN PROLINE HYDROXYLASE

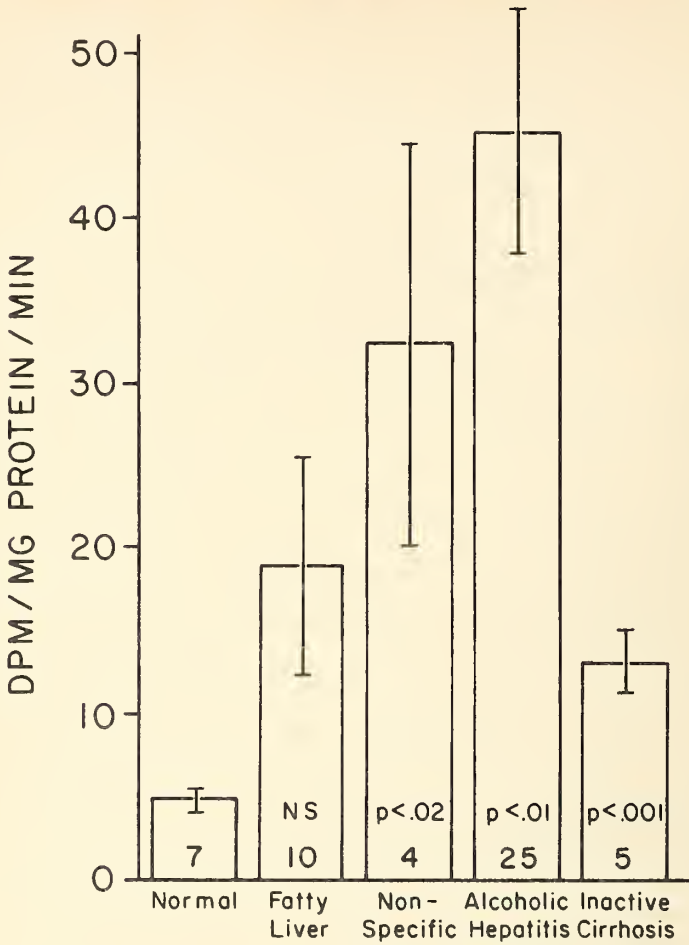


Figure 1. Activities of protocollagen proline hydroxylase in patients with various types of alcoholic liver disease as compared with normal controls. The values are expressed as means \pm SEM. The number of subjects in each group, and the probability levels of a difference in values from the normal controls are indicated inside the bars.

The mean activity of hepatic protocollagen proline hydroxylase in the 7 non-alcoholic control patients was 4.87 ± 0.69 d.p.m./mg protein/min (Figure 1), or 0.52 ± 0.06 d.p.m. $\times 10^3$ /g liver wet weight/min. Patients with alcoholic hepatitis had the highest activity of protocollagen proline hydroxylase with mean values of 45.18 ± 7.44 d.p.m./mg protein/min ($p < .01$) or 3.67 ± 0.62 d.p.m. $\times 10^3$ d.p.m./g liver wet weight/min ($p < .02$). Also markedly increased were the mean activities of protocollagen proline hydroxylase in patients with non-specific changes on liver biopsy; 32.24 ± 12.36 d.p.m./mg protein/min ($p < .02$) or 3.44 ± 1.03 d.p.m. $\times 10^3$ /g liver wet weight/min ($p < .01$). The values in patients with inactive cirrhosis while much less increased to 13.38 ± 1.72 d.p.m./mg protein/min, or $1.53 \times 10^3 \pm 0.16$ d.p.m. $\times 10^3$ /g liver wet weight/min, were nevertheless significantly different from control values at $p < .001$. On the other hand, patients with fatty infiltration of the liver had mean activities of 18.91 d.p.m. ± 6.51 /mg protein/min, or 1.88 d.p.m. $\pm 0.67 \times 10^3$ /g liver wet weight/min, which were not significantly different from control values.

In the patients with alcoholic hepatitis the highest activities of hepatic procollagen proline hydroxylase were found in those with the most extensive fibrosis on liver biopsy (Figure 2). On the other hand, no correlation was found between the activity of the enzyme and the degree of fatty infiltration (+1 in two, +2 in three, +3 in three, and +4 in two of the 10 patients with fatty liver).

PROTOCOLLAGEN PROLINE HYDROXYLASE

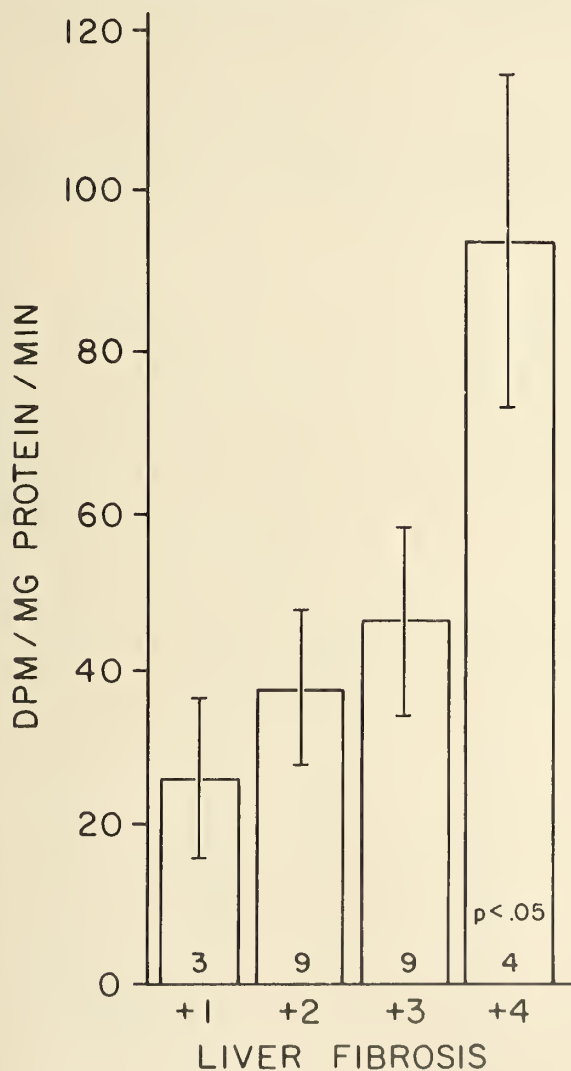


Figure 2. Comparison of the activities of hepatic procollagen proline hydroxylase in the 25 patients with alcoholic hepatitis. The values are expressed as means \pm SEM. The histological grading of fibrosis is indicated in the text. The number of subjects in each group, and the probability levels of a difference in values from those found with +1 (minimal) fibrosis are indicated inside the bars.

The urinary excretion of total hydroxyproline in the normal controls was 34.7 ± 3.61 mg/24 hours (Figure 3). The patients with alcoholic hepatitis had a significant elevation of total urine hydroxyproline to a mean value of 58.4 ± 5.6 mg/24 hours ($p < .05$). The mean values in the other groups of patients were not significantly different from the normal value. They were as follows: fatty infiltration of the liver, 34.8 ± 3.6 mg/24 hours; non-specific changes, 34.4 ± 6.9 mg/24 hours; and inactive cirrhosis, 46.6 ± 4.5 mg/24 hours. No correlation was found between the degree of fibrosis and the total urinary excretion of hydroxyproline in the patients with alcoholic hepatitis.

The urinary excretion of free hydroxyproline consisted of only a 1.0 to 3.4% fraction of the total excretion of hydroxyproline in all the determinations, with no difference in values between the various groups of alcoholic patients and the normal controls.

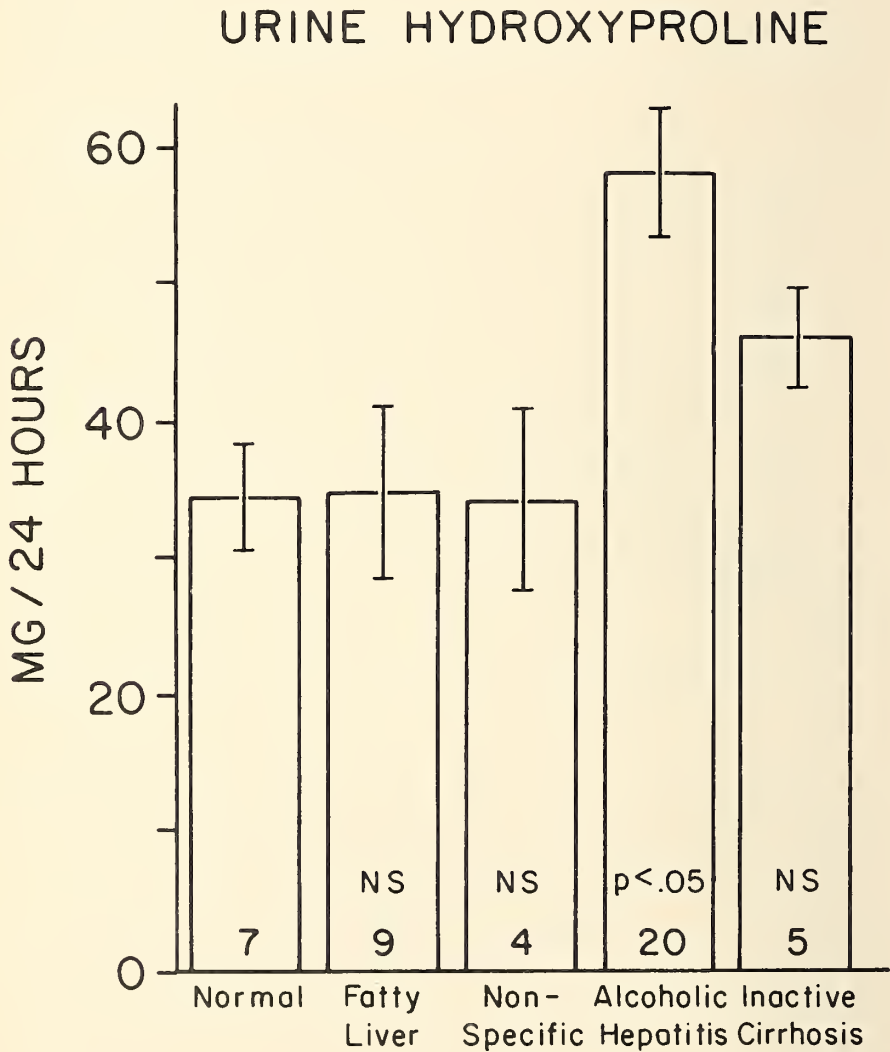


Figure 3. Urinary excretion of hydroxyproline in patients with various types of alcoholic liver disease as compared with normal controls. The values are expressed as means \pm SEM. The number of subjects in each group, and the probability levels of a difference in values from the normal controls are indicated inside the bars.

DISCUSSION

The finding of the greatest increase in the activity of hepatic procollagen proline hydroxylase in the alcoholic patients with the lesion of alcoholic hepatitis is not surprising, since, while the stimulus for activation of fibroblastic activity is not known, most studies indicate that necrosis and inflammation, rather than steatosis, is the important factor in the pathogenesis of cirrhosis (Alterman, 1954; Shorter and Baggenstoss, 1959). An exception to this view is the suggestion by Hartroft (1954) that fatty infiltration with the formation and subsequent rupture of fatty cysts with condensation of the parenchymal and stromal remnants results in the formation of fibrous trabeculae. However, increases in both synthesis of Kupffer cells and undifferentiated mesenchymal or ductular cells in connective tissue stroma were shown in liver biopsy specimens with evidence of necrosis, but not in those with fatty infiltration alone (Leevy, tenHove and Howard, 1964). Also increased incorporation of ^3H -proline into collagen in the presence of ethanol *in vitro* was found in liver biopsy samples from patients with alcoholic hepatitis and cirrhosis but not in those with fatty liver (Chen and Leevy, 1972). Furthermore, the experimental orotic acid induced fatty infiltration of the liver without necrosis does not progress to cirrhosis (Handschumacher, Creasey, Jaffe, Pasternak and Hankin, 1960). The greater increase in the activity of hepatic procollagen proline hydroxylase in the alcoholic patients with the more extensive fibrosis is consistent with the observation in animals that in carbon tetrachloride induced hepatic fibrosis there is a correlation between the capacity to synthesize collagen and the content of collagen (Rojkind and Diaz de Leon, 1970). This correlation however, was only found in the presence of hepatic necrosis and inflammation, since in patients with inactive cirrhosis the activity of procollagen proline hydroxylase although higher than found in controls was much lower than found in the patients with alcoholic hepatitis.

The significant increase in the activity of procollagen proline hydroxylase in the alcoholic patients with non-specific changes on liver biopsy such as periportal inflammation or mild focal parenchymal necrosis was an unexpected finding. While these non-specific changes may represent either an early or a late stage in the spectrum of alcoholic hepatitis, studies of the enzyme activity in a variety of patients with non-alcoholic types of liver disease will be required to clarify the above findings.

The increases in the urinary excretion of hydroxyproline in the patients with alcoholic hepatitis, suggest that the increased collagen synthesized and deposited in the liver of these patients is also degraded faster. While the mean urinary excretion of hydroxyproline was found to be increased above normal in 31 patients with cirrhosis of the liver by Kratzch (1969), our results in 5 patients show only a slight increase which is not statistically significant ($0.5 < p < 0.1$). This difference in results may be related to the small number of determinations in our study or to the inclusion of some cases with evidence of alcoholic hepatitis in the former study and therefore requires further investigation.

Although the urinary excretion of hydroxyproline has been shown to be increased after the ingestion of gelatin and collagen-containing foods (Prockop and Sjoerdsma, 1961), and decreased after starvation in infants (Picon, Alleyne and Seakins, 1965), it is unlikely that these factors influenced our results since the patients with alcoholic hepatitis had a similar history of poor dietary intake prior to admission, and were placed on a similar hospital diet after admission, as did the other alcoholic patients studied.

In conclusion, the observed increases in the activity of hepatic procollagen proline hydroxylase and in the urinary excretion of hydroxyproline in patients with alcoholic

hepatitis suggest increased hepatic collagen turnover (both increased synthesis and degradation) in this condition. These findings in alcoholic hepatitis contrasted with the lack of similar changes in patients with fatty infiltration of the liver are consistent with the clinical observations that alcoholic hepatitis is a precursor of cirrhosis while fatty infiltration of the liver is basically a reversible condition.

SUMMARY

The activity of hepatic procollagen proline hydroxylase and the urinary excretion of hydroxyproline were determined as parameters reflecting liver collagen synthesis and degradation respectively, in forty-four patients with alcoholic liver disease. The activity of procollagen proline hydroxylase was significantly increased above normal controls in the alcoholic patients with liver biopsies showing alcoholic hepatitis, non-specific changes, and inactive cirrhosis, but not in those with fatty infiltration of the liver. The highest activities of procollagen proline hydroxylase were found in the patients with alcoholic hepatitis, and among these patients, the enzymatic activity was higher in those with the most extensive liver fibrosis. The urinary excretion of hydroxyproline was increased above normal only in the patients with alcoholic hepatitis.

These studies show that among the types of liver disease occurring in alcoholic patients, alcoholic hepatitis is associated with the greatest increase in collagen turnover (both increased synthesis and degradation). This finding is consistent with the clinical observation that the lesion of alcoholic hepatitis is a precursor of cirrhosis of the liver.

ACKNOWLEDGEMENTS

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The Lymphocyte and Liver Disease of the Alcoholic

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Natural history studies indicate that alcoholic hepatitis is a precursor of cirrhosis, and that these lesions occur in 10 to 30 per cent of alcoholics (Leevy, 1967). Both have been attributed to metabolic or toxic effects of ethanol, nutrient deficits, or to a combination of these factors. Alcoholic hepatitis does not regularly lead to cirrhosis although each succeeding episode increases the potential for its development. The time required for the transformation of alcoholic hepatitis to cirrhosis with continued alcoholism may vary from several months to several years (Leevy, 1962).

Our observations indicate that the appearance of alcoholic hepatitis cannot be related to the amount, duration, or type of alcohol ingested, although alcoholic hepatitis has only been produced in human volunteers fed enormous quantities of ethanol. There is also no correlation between occurrence of alcoholic hepatitis and previous dietary habits, or stigmata of nutritional deficiency (Leevy, 1967). Ethanol or starvation causes identical disaggregation of ribosomes from polysomes of the hepatocyte (Theron and Lienberg, 1963; Rothschild, Oratz, Mongelli and Schreiber, 1971). It has also been shown that fat, hyalin, and fibrosis, indistinguishable from that seen in alcoholics, may occur as a result of altered body nutriture imposed by jejuno colic or jejuno ileal bypass in non-alcoholics (Sorrell, Baker and Leevy, 1971; Peters and Reynolds, 1973).

The varying susceptibility of alcoholics to pancreatitis and cardiomyopathy, despite the same pattern of alcohol and food intake, suggests that constitutional or genetic factors are key determinants of the type of tissue injury. Occasional hypersensitivity reactions following ingestion of alcoholic beverages makes it desirable to evaluate the role of altered immunologic reactivity in development of cirrhosis. The suggestion that immune mechanisms may contribute to alcoholic hepatitis is supported by the progressive

shortening of the interval required for reoccurrence of this lesion after resumption of alcoholism.

Liver disease of the alcoholic provides a unique opportunity to study immunologic abnormalities in hepatic disorders: (a) malnutrition, which is ever present in the alcoholic with liver disease, is a key determinant of immunocompetence (McFarlane and Hamid, 1973); (b) acetaldehyde and congeners in alcoholic beverages can depolymerize protein, thus providing the setting for antigenic alterations; (c) morphologic changes in alcoholic hepatitis simulate a Type III Arthus reaction. Unlike patients with chronic active hepatitis or biliary cirrhosis, surveys of alcoholics with uncomplicated fatty liver, alcoholic hepatitis, or cirrhosis reveal only non-specific alterations of serum immunoglobulins and a low incidence of mitochondrial, smooth muscle and antinuclear antibodies (Akdamar, Epps, Maumus and Sparks, 1972; Doniach and Walker, 1972). On the other hand, studies in our laboratory indicate that significant changes in cell-mediated immunologic reactivity occur in alcoholic hepatitis and may contribute to the development of chronic liver disease (Doniach and Walker, 1972).

BACKGROUND

Serum immunoglobulins and circulatory antibodies, lymphocyte transformation and migration inhibition factor (MIF) production have been used to study immunologic reactivity in liver disease. The concept that host (immunologic) factors initiate and perpetuate liver injury was proposed in the early part of the twentieth century (Fiessinger, 1908). Recent advances in basic knowledge and development of clinical techniques for study of immunologic reactivity in man now permits objective evaluation of this hypothesis. Lymphocytes produce antibodies as well as a variety of factors essential for host

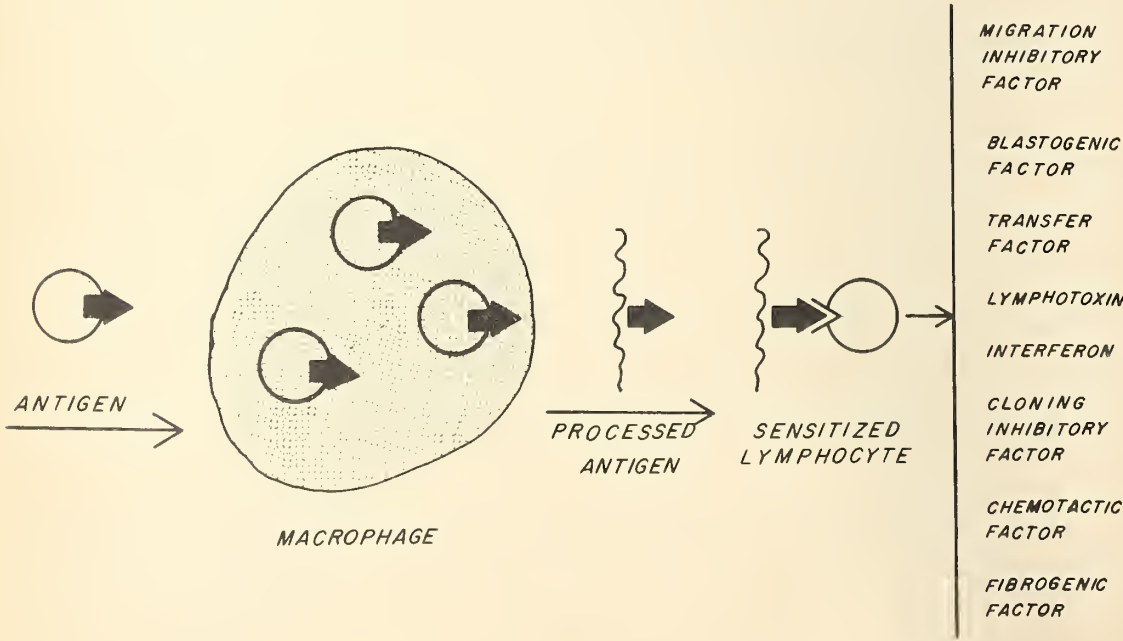


Figure 1. Factors elaborated during cell-mediated immunologic reactivity.

protection. Bursal-dependent (B-cell) lymphocytes produce antibody, whereas, thymus-dependent (T-cell) lymphocytes elaborate the other factors (Fig. 1). These two types of lymphocytes may be quantified or separated based on morphology (Lin, Cooper and Wortis, 1973), surface content (Papamichail, Brown and Holborow, 1971), and/or affinity for sheep red blood cells (Wybran, Curr and Fudenburg, 1972).

EVALUATION OF LYMPHOCYTE REACTIVITY

Liver injury has been attributed to humoral or antibody-mediated reactivity, and more recently to cellular immunity. Interest in cellular reactivity followed the wide use of assays for blastogenic factor (Bach and Hirschhorn, 1965), and MIF (Saborg and Bendixen, 1967) in a variety of immunologic disease states. A new lymphocyte factor, "fibrogenic factor", has been discovered (Chen, Zetterman and Leevy, 1973) which may have far reaching implications in pathogenesis of chronic liver disease.

Lymphocytes are cultured using a modification of the method of Bach and Hirschhorn (1965), with addition of various test agents in studies of blastogenic factor. Cultures are incubated at 37°C under 5 per cent carbon dioxide and harvested after 3 to 5 days. Transformation of cultured lymphocytes is assessed by studies of the incorporation of tritiated thymidine into DNA. Results are expressed by stimulation indices derived by dividing control values into test values. Using this method, lymphocytes from normal subjects exhibit a stimulation index of 35 or more when the mitogen phytohemagglutinin (PHA-M) is added to the culture.

Direct evaluation of MIF is achieved by a modification of the method of Soborg and Bendixen (1967). Heparin-free capillary tubes are filled with a leukocyte suspension obtained from the patient under study. The suspension is centrifuged, cut beneath the cell fluid interphase, and placed in a Lexy culture chamber. Control chambers are filled with complete media, and experimental chambers with media plus test agents. The cells are allowed to migrate for 18 hours at 37°C under 5 per cent carbon dioxide atmosphere, and the area of migration determined by planimetry. Results are expressed as migration index derived by dividing test areas by the control. The index is the reciprocal of MIF production.

Elaboration of fibrogenic factor by sensitized lymphocytes is determined by adding the cell-free supernatant of lymphocytes cultured in the presence of antigen in L929 fibroblast cultures or incubated liver biopsies which are subsequently pulsed with tritiated proline (Chen *et al.*, 1973). Collagen is isolated and the incorporation of labeled hydroxyproline is utilized as an index of collagen synthesis (Juva and Prockop, 1966). The activity of supernatants from test cultures is compared to that of controls. PHA-stimulated lymphocytes consistently release this factor and produce a 3-fold or greater increase in collagen synthesis.

IMMUNOLOGIC REACTIVITY IN NON-ALCOHOLIC LIVER DISEASE

Schistosomiasis

Terminal triaditis, hepatic granulomata, and pipestem fibrosis due to schistosomiasis are the best examples of a liver lesion due to delayed-type hypersensitivity (Warren, 1972). Host immunologic response to schistosome ova has been demonstrated by Warren to be a

key event in pathogenesis of hepatic schistosomiasis (Domingo, Cowan and Warren, 1967). An intact immune system is clearly needed, since thymectomized mice infected with schistosomiasis develop multiple abscesses in contrast to the multiple granulomata seen in mice with normal immune systems. Studies in the guinea pig show a specific relation of granuloma formation to delayed skin response and *in vitro* reactivity of lymphocytes to soluble antigens extracted from schistosome eggs. Granulomatous hypersensitivity is induced by secretion of soluble antigens through ultramicroscopic pores of eggshells. Immunological tolerance to granuloma formation by intact schistosome eggs can be induced by this antigen (Domingo *et al.*, 1967).

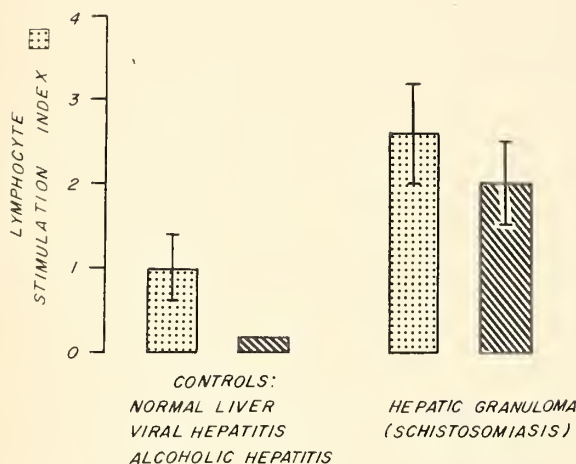


Figure 2. Influence of cercarial antigen on stimulation index of lymphocytes from patients with and without schistosomiasis.

Cercarial antigen added to lymphocytes from patients with hepatic granulomata due to schistosomiasis *mansoni* results in significant increase in the lymphocyte stimulation index with a parallel increase in MIF production (Fig. 2). Supernatants from these lymphocytes cause a 2-3-fold increase in incorporation of hydroxyproline into collagen of fibroblast cultures. Autologous liver that contains schistosomal granuloma will produce a similar effect. In our experience, neither antimony nor removal of worms by filtration alters cell-mediated immunologic reactivity in this disease.

Viral Hepatitis B

Discovery of an antigenic marker for viral hepatitis B has permitted a wide range of studies on immunologic reactivity in this disease. Acquired immunity to viral infections has traditionally been considered to be a function of humoral antibodies. Indeed, detectible antibody is usually demonstrable following hepatitis B infection (Barker, Peterson, Shulman and Murray, 1973), and peaks at a time of maximal hepatocellular injury (Sherlock, 1972). This suggests antigen-antibody complexes may produce liver injury; however, the presence of normal circulating levels of complement makes this unlikely (Fox, Dudley and Sherlock, 1971). Direct viral cytotoxicity has been implicated as a cause for hepatocyte necrosis; against this postulate is the very high incidence of hepatitis B within hepatocytes of patients who are antigen carriers and who exhibit normal liver morphology (Reinicke, Dybkjaer, Poulsen, Banke, Lylloff and Nordenfelt, 1972).

Circumstantial evidence suggests cellular immunoreactivity is the chief determinant of the course of hepatitis B infection (Dudley, Fox and Sherlock, 1971; Zetterman, Tamburro and Leevy, in press). During an acute infection with the virus of hepatitis B, PHA responsiveness — a presumed indication of overall T-cell reactivity — is markedly suppressed and returns to normal with healing (Zetterman *et al.*, in press) (Fig. 3). Carriers of hepatitis B antigen, and patients with chronic persistent or chronic active hepatitis may exhibit continued suppression of T-cell responsiveness (Giustino, Dudley and Sherlock, 1972), suggesting that a depression of cellular immunity contributes to development of chronic injury. Thus, individuals who contract hepatitis B while receiving immunosuppressive therapy tend to remain antigen positive and to develop chronic liver disease (Aronoff, Gault, Huang, Lal, Wu, Moinuddin, Spence and MacLean, 1973). It seems probable that antigenic determinants of the virus are incorporated into covering membranes of the infected hepatocyte. T-cell sensitization induced by circulating antigen would appear to evoke hepatocyte damage or necrosis.

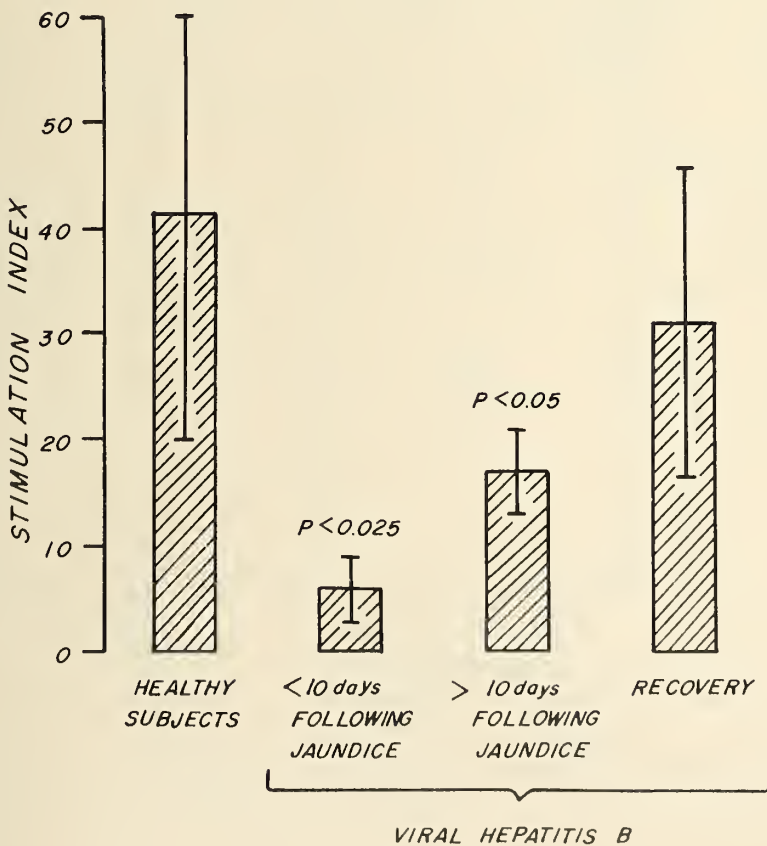


Figure 3. PHA response of cultured lymphocytes from patients with viral B hepatitis.

IMMUNOLOGIC REACTIVITY IN LIVER DISEASE OF THE ALCOHOLIC

Alcoholics with liver disease exhibit a significant decrease in circulating "T" cells and a marked variation in the stimulation index after addition of PHA to cultured lymphocytes (Zetterman and Leevy, in press). Patients with alcoholic hepatitis regularly exhibit a reduction in response (Fig. 4). Investigations were undertaken to determine the cause for lack of response in individual patients. It was found that in some instances a nutritious diet given over a period of 7 to 10 days led to a restoration of PHA responsiveness to expected values. Folate and vitamin B₁₂-depleted patients who had a decrease in *in vitro* hepatic or gastric mucosal DNA synthesis also regularly had a decrease in lymphocyte responsiveness. In addition, two thermolabile plasma factors that interfere with PHA responsiveness have been discovered, one in alcoholics with cirrhosis (Hsu and Leevy, 1971), and another in patients with chronic liver disease and uremia (Chen and Leevy, 1973). The uremic factor is also dialyzable and has been shown to interfere with *in vitro* hepatic DNA synthesis (Chen and Leevy, 1973).



Figure 4. PHA response of cultured lymphocytes from patients with alcoholic liver disease.

Addition of hepatitis B antigen to lymphocytes from patients with alcoholic hepatitis may also evoke an increase in the stimulation index and MIF production, although such persons are antigen and antibody negative and give no history to suggest that they have had viral hepatitis previously (Zetterman and Leevy, in press). Autologous liver homogenate added to lymphocyte cultures from alcoholics with normal liver, fatty

liver, or inactive cirrhosis have no influence on the stimulation index or MIF production. In contrast, a significant increase in the stimulation index occurs with addition of autologous liver to lymphocytes of patients with alcoholic hepatitis equivalent to that seen in granuloma due to schistosomiasis or chronic active hepatitis (Fig. 5).

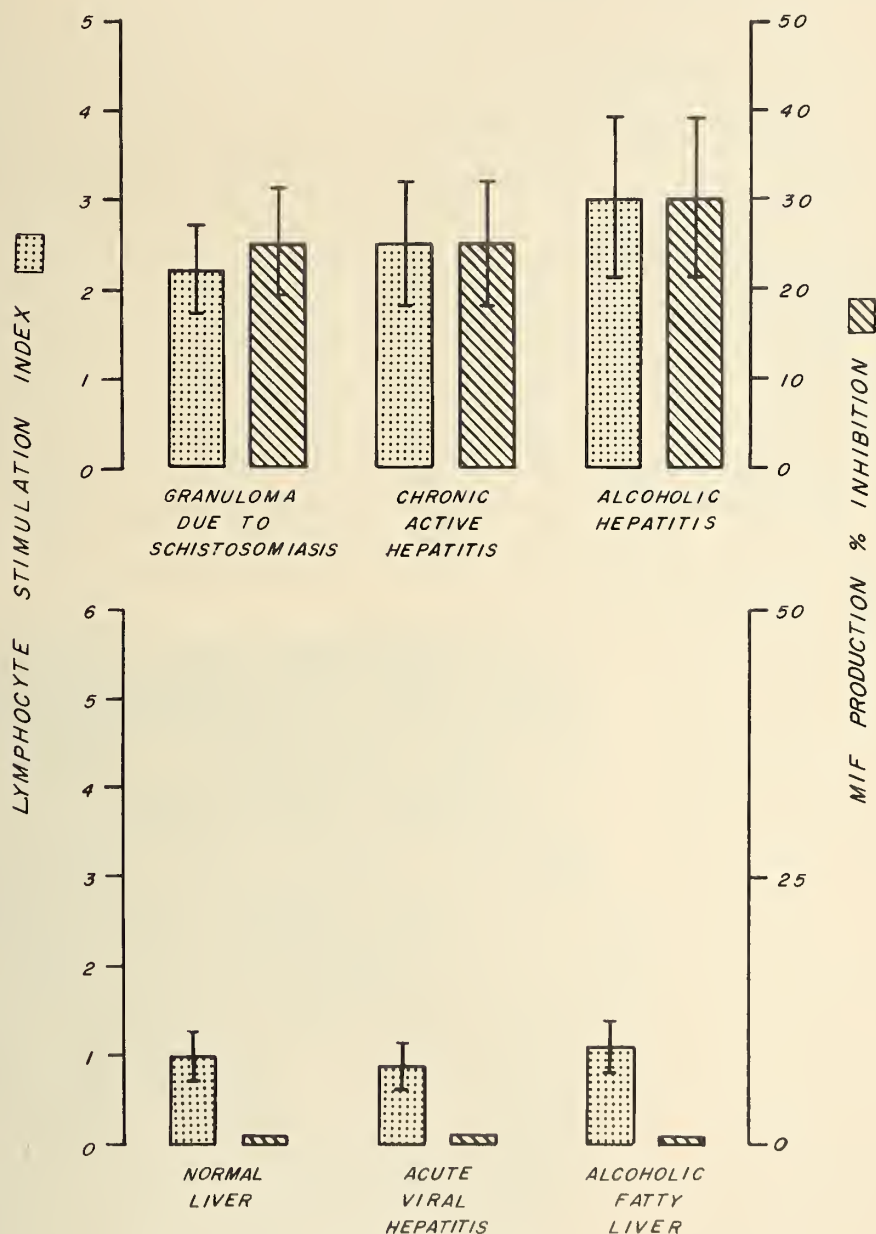


Figure 5. Response of cultured lymphocytes to autologous liver from patients with various hepatic disorders. Reproduced from Leevy, Liver Regeneration in Man, Charles C. Thomas, 1973.

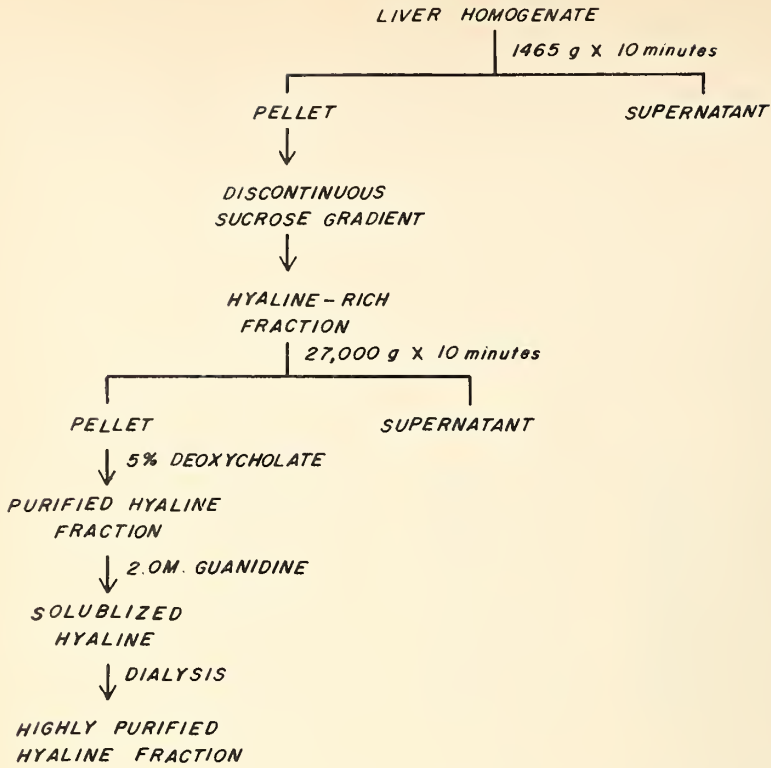


Figure 6. Procedure for preparation of purified alcoholic hyaline (French).

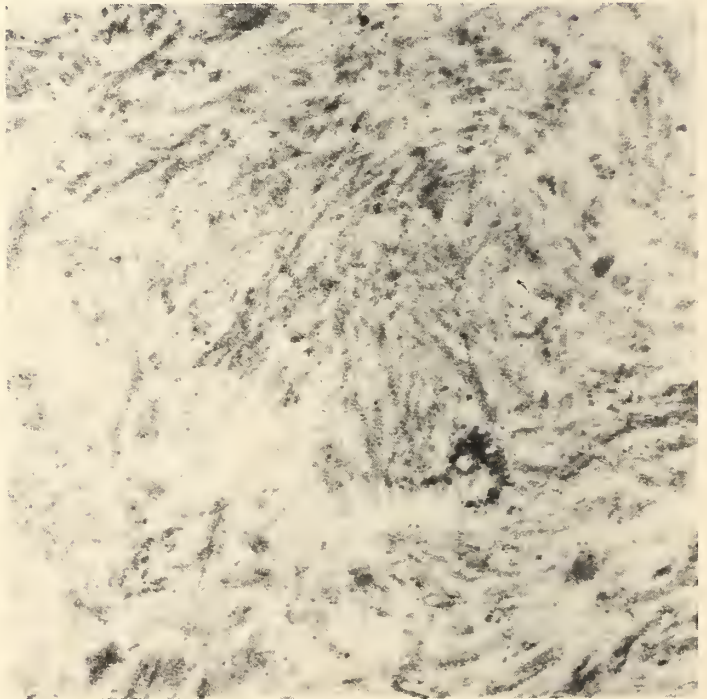


Figure 7. Electronmicrograph of hyalin isolated from patient dying of alcoholic hepatitis. Note randomly oriented fibrillar structure. Reproduced from Leevy, Evaluation of Liver Function in Clinical Practice, Lilly Research Laboratories, 1974.

Alcoholic Hyalin and Immunologic Reactivity

A systematic investigation has been undertaken to determine the reason autologous liver stimulates replication of lymphocytes from patients with alcoholic hepatitis. Each of the patients in the study group exhibited alcoholic hyalin with leukocytic infiltration, with or without steatosis or fibrosis. It was suspected that the alcoholic hyalin might be responsible for the encountered reaction, since there is a concomitant reduction of lymphocyte reactivity and histologic evidence of hyalin.

Hyalin was isolated for in depth studies of lymphocyte reactivity utilizing the French technique (French, Ihrig and Norum, 1972). Homogenates of postmortem liver were layered over a discontinuous sucrose gradient centrifuged at 99,000 g and incubated with deoxycholate; subsequently, the purified hyalin was dissolved in guanidine (Fig. 6). This material studied under the electron microscope revealed a randomly oriented fibrillar structure, characteristic of Type II hyalin (Fig. 7). Addition of the purified hyalin fraction to lymphocytes from patients with alcoholic hepatitis caused a significant increase in production of migration inhibition factor, but had no effect on lymphocytes from normal subjects or patients with fatty liver (Zetterman, Luisada-Opper and Leevy, 1973) (Fig. 8). Moreover, lymphocytes from patients with chronic active hepatitis or cholestasis which exhibited hyalin on biopsy showed no response.

Purified alcoholic hyalin had a variable effect on incorporation of tritiated thymidine into DNA of lymphocytes (stimulation index). Lymphocytes with a normal response to PHA exhibited increased stimulation index and MIF after purified hyalin was

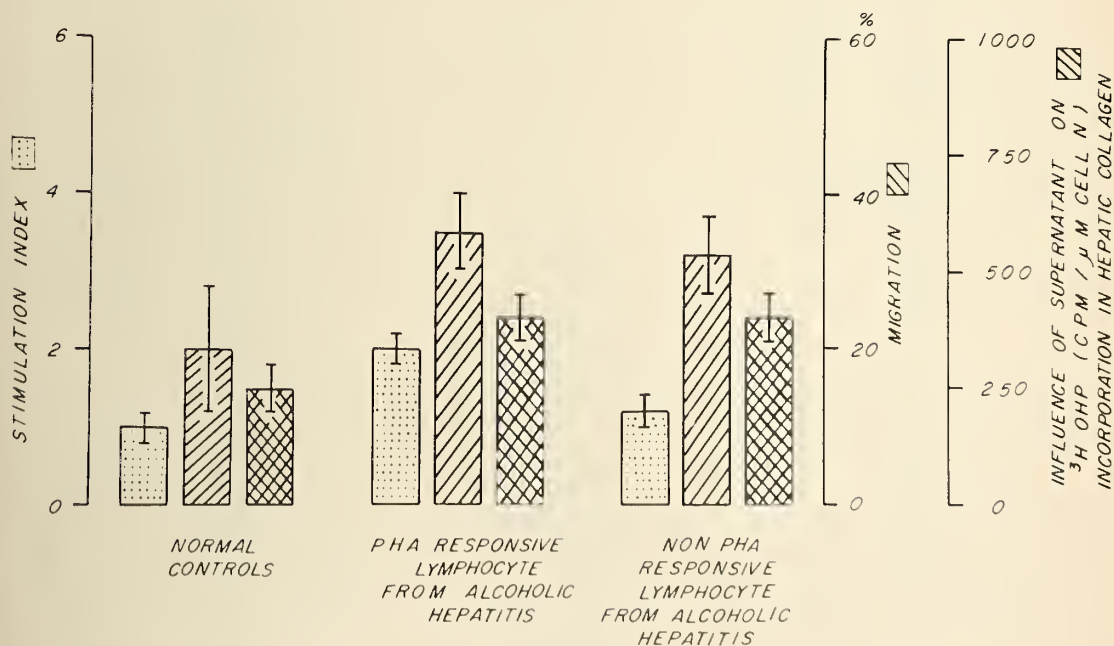


Figure 8. Response of cultured lymphocytes from controls and patients with alcoholic hepatitis to purified alcoholic hyalin. This material evoked an increase in stimulation index and migration inhibition in PHA responsive lymphocytes from patients with alcoholic hepatitis. The supernatant from the hyperactive lymphocytes caused a significant increase in incorporation of ^3H OPH into collagen.

added (Fig. 8). Lymphocytes with diminished response to PHA had a negligible S.I. but normal MIF response to purified hyalin. When the supernatant of alcoholic hyalin-stimulated lymphocytes was added to cultured fibroblasts, there was a significant increase in the incorporation of tritiated hydroxyproline into collagen (Fig. 8). Supernatants from lymphocyte cultures from patients with chronic active hepatitis or schistosomiasis to which purified hyalin was added were without effect in collagen synthesis. The fibrogenic supernatant did not significantly alter either incorporation of tritiated thymidine into DNA or tritiated leucine into protein.

Ethanol and Immunologic Reactivity

The central role of ethanol and its metabolites in pathogenesis of alcoholic hepatitis prompted addition of these agents to lymphocyte cultures at various dose levels (Sorrell and Leevy, 1972). Lymphocytes were killed when ethanol was added to cultures in quantities of more than 4 mg per ml of culture, or acetaldehyde was added in quantities of over 10 μ g per ml per culture. In contrast, less than 2 mg per ml of ethanol or less than 5 μ g per ml of acetaldehyde caused a 2- to 3-fold increase in transformation of lymphocytes from patients with alcoholic hepatitis. These additives had no effect on alcoholics

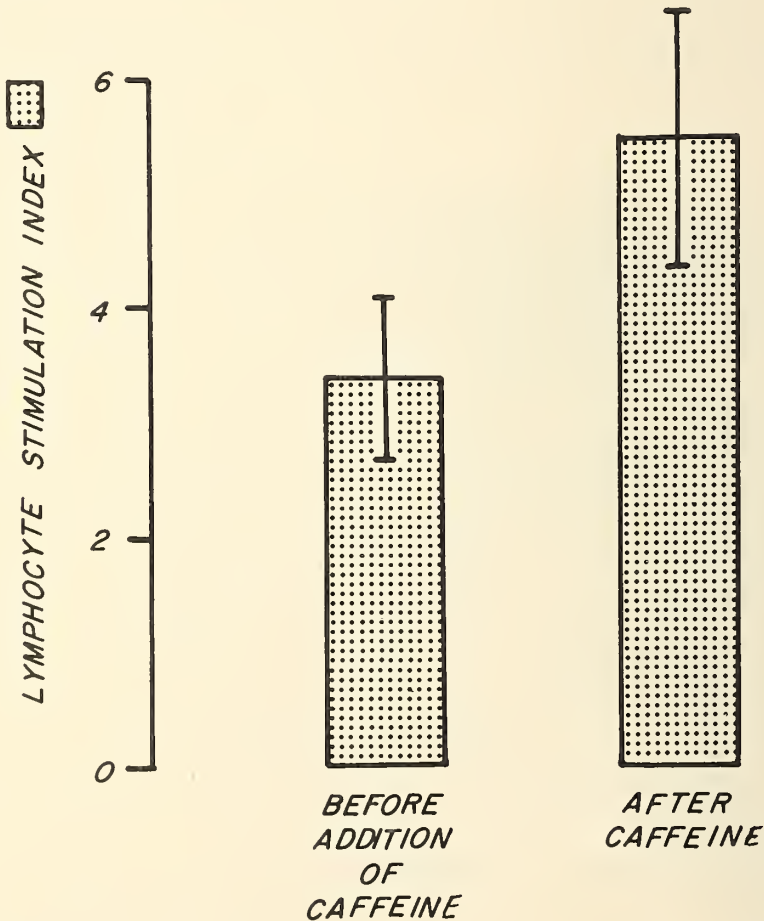


Figure 9. Influence of acetaldehyde (5 μ g per ml culture) and acetaldehyde plus caffeine on stimulation index of lymphocytes from patients with alcoholic hepatitis.

with fatty liver or inactive cirrhosis. Serial studies of lymphocyte cultures indicated ethanol was largely oxidized to acetaldehyde, suggesting the latter was responsible for the observed stimulating effect. Since there is no proof that ethanol or acetaldehyde act as direct antigens, it was decided to evaluate the means whereby observed lymphocyte stimulation occurs. Studies were first conducted to determine if they activated the cyclic AMP system. Addition of the phosphodiesterase inhibitor, caffeine, to the acetaldehyde-lymphocyte system causes an increase in stimulation index, suggesting cyclic AMP is released (Fig. 9). Direct measurements are in progress. *In vitro* reactivity to ethanol or acetaldehyde is increased if one of these agents is added with autologous liver homogenate or purified alcoholic hyalin to cultured lymphocytes.

Autoradiographic studies of liver biopsies from patients with alcoholic hepatitis incubated in tritiated thymidine show a marked proliferation of mesenchymal cells (Leevy, 1966). If one adds 0.5 to 2 mg per ml of ethanol to the incubation media, there is a significant reduction in DNA synthesis. On the other hand, addition of this amount of ethanol evokes a marked increase in incorporation of tritiated hydroxyproline into salt-soluble and salt-insoluble collagen in alcoholic hepatitis (Chen and Leevy, in press). This effect of ethanol on collagen synthesis is not observed in fatty liver or inactive cirrhosis in alcoholics.

Therapeutic Aspects

Improvement in liver function and morphology in patients with alcoholic hepatitis is associated with an increase in PHA responsiveness, while progressive liver failure is associated with a persistent reduction in this parameter. A decrease in lymphocyte response to PHA is associated with a reduction in *in vitro* hepatic DNA synthesis, while an increase in lymphocyte responsiveness is accompanied by a parallel increment in hepatic DNA synthesis. Exaggerated responsiveness of lymphocytes obtained from patients with alcoholic hepatitis to ethanol, alcoholic hyalin, or autologous liver disappears with clinical or histologic improvement. Patients with alcoholic hepatitis and increased lymphocyte reactivity who continue to consume ethanol exhibit progressive liver cell destruction with development of cirrhosis within an 11-month period after resumption of alcoholism (Sorrell and Leevy, 1972).

The reversible reduction in lymphocyte reactivity with improvement in liver function provides a basis for treatment of the malnourished alcoholic with hepatitis by diet and appropriate nutritional supplements needed to restore normal DNA synthetic capacity. Theoretically, corticosteroid-induced inhibition of an exaggerated immune response could minimize tissue damage and facilitate recovery. However, data from controlled studies suggest adrenal steroids do not accelerate or facilitate recovery from this condition (Porter, Simon, Pope, Volwiler and Fenster, 1971; Blitzler, Mutchwick, Joshi, Phillips, Fessel and Conn, 1973; Campra, Hamlin, Kirshbaum, Oliver, Redeker and Reynolds, 1973).

THEORETICAL CONSIDERATIONS

Investigations of immunologic reactivity in liver disease have introduced a new concept in the effort to identify factors which cause one alcoholic to develop cirrhosis while another with identical alcohol and food patterns remains free of this disease. It is possible

that ethanol or acetaldehyde activates the lymphocyte system by physico-chemical action on the cell and alters liver protein to evoke increased production or decreased dispersal of hyalin (French and Davies, 1974), demonstrated to be characteristic of alcoholic hepatitis. In this event, the all important T-cell reactivity would depend on nutritional and genetic factors as well as the amount and type of alcoholic beverages ingested.

The finding that nutrient deficiency imposed in non-alcoholics by a jejuno ileal bypass for control of obesity causes deposition of hyalin (Sorrell *et al.*, 1971; Peters and Reynolds, 1973) suggests nutrient deficits, as yet unidentified, are responsible. Metabolic or toxic effects of ethanol may increase the underlying abnormality. Ethanol and hyalin may be additive in either increasing lymphocyte reactivity or stimulating collagen deposition (Fig. 10). Nutrient deficiency, by interfering with collagenase activity, could prevent reabsorption of collagen and predispose to progressive fibrosis.

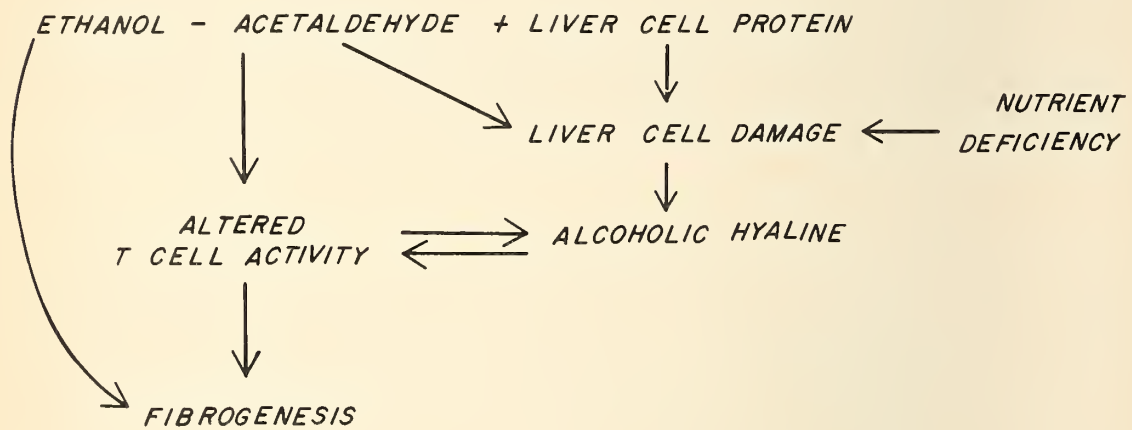


Figure 10. Possible interrelationships of alcoholic hyaline, altered T-cell activity, and fibrogenesis.

Our demonstration that sensitized lymphocytes react to hyalin and produce a fibrogenic factor makes it desirable to explore ways to utilize this information in prevention and treatment of chronic liver disease of the alcoholic. It is desirable to determine the chemical composition of hyalin and develop immunologic tests to screen alcoholics for early reversible phase disease. Additional controlled studies are necessary to explore therapeutic implications of lymphocyte reactivity. An effort should now be made to determine the influence of corticosteroids, immunosuppressive agents, or colchicine on immunologic reactivity, hyaline deposition, or absorption, and fibrogenesis in alcoholic hepatitis (Leevy and Zetterman, 1973).

SUMMARY AND CONCLUSIONS

1. Studies of cell-mediated immunologic reactivity in liver disease of the alcoholic indicate that there is frequently a decrease in immunocompetence as reflected in a reduction of lymphocyte responsiveness to PHA. This is attributable to nutrient deficiency, the presence of circulating antagonists or lymphocyte injury.

2. PHA responsive lymphocytes from patients with alcoholic hepatitis exhibit a significant increase in transformation when autologous liver or a purified extract of alcoholic hyalin is added. These additives cause an increase in MIF production in both PHA responsive and non-responsive lymphocytes. The supernatant from such lymphocytes evokes an increase in the incorporation of hydroxyproline into collagen.

3. It is postulated that the lymphocytes play a key role in pathogenesis of cirrhosis in alcoholics. Nutrient deficits alone or ethanol plus nutrient deficits cause increased production or decreased dispersal of hyalin. Hyalin leads to T-cell hyperactivity with release of a fibrogenic factor which may contribute to the development of hepatic fibrosis.

4. Treatment of the alcoholic with liver injury should include measures to restore normal lymphocyte reactivity and increase hepatic DNA synthetic capacity by provision of deficient nutrients and removal of circulating antagonists. Equal benefit may occur from therapy designed to eliminate or modify factors responsible for exaggerated immunologic response.

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Alcohol and the Liver: Transition from Metabolic Adaptation to Tissue Injury and Cirrhosis

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INTRODUCTION

The present article will focus primarily on an analysis of the progress achieved in recent years. Several previous reviews summarized in detail the evidence which had accumulated to indicate that, independent of nutritional deficiencies, alcohol itself can be incriminated in the pathogenesis of liver injury, as evidence by fat accumulation and striking ultrastructural changes (Lieber, 1966; 1968). The hepatotoxicity of ethanol has been substantiated further in the rat (DeCarli and Lieber, 1967; Gordon and Lough, 1972), in dogs (Chey, Kosay, Spilet and Lorber, 1971), in the baboon (Lieber, DeCarli, Gang, Walker and Rubin, 1972), and in man, both in alcoholic (Lane and Lieber, 1966; Lieber and Rubin, 1968) and nonalcoholic (Rubin and Lieber, 1968a; Wiebe, Lundquist and Belfrage, 1971) volunteers. The pathologic changes included not only fatty liver, but also striking structural changes, to be discussed subsequently. Supplementation of the diet with large amounts of protein, minerals and vitamins (including choline chloride) had no apparent effect on the alcohol-induced lesion in man (Lieber and Rubin, 1968; Rubin and Lieber, 1968a) and did not fully prevent the effects in the rat (Lieber and DeCarli, 1966). A severe deficiency in proteins and in other lipotropic factors, however, potentiated the effect of ethanol, at least in animals (Lieber, Spritz and DeCarli, 1969; Takeuchi, Takada, Kanayama, Ohara and Okumura, 1969).

Another major point of emphasis of previous reviews (Lieber and Davidson, 1962; Lieber, 1969; 1972) was that of the importance of the increase in NADH/NAD ratio produced by the oxidation of ethanol in the liver and the immediate concomitant metabolic alterations reflecting this redox change, some of which are illustrated in Figure 1. The studies of various metabolic alterations which can be considered as direct conse-

METABOLIC EFFECTS OF ETHANOL IN THE HEPATOCYTE

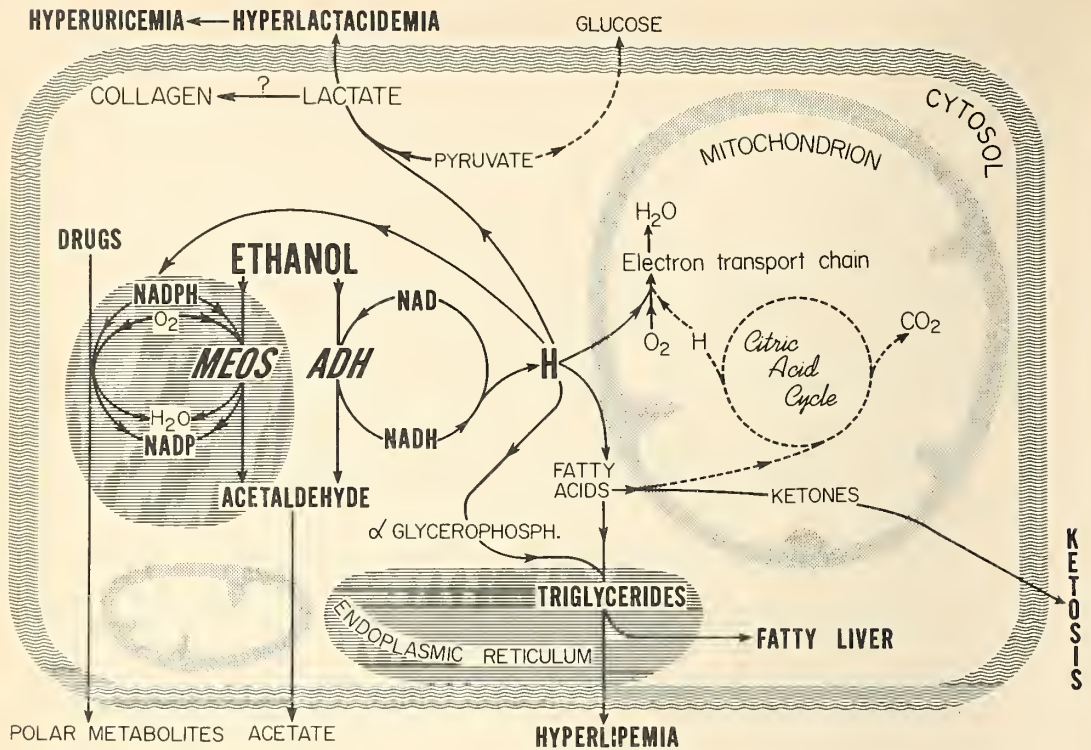


Figure 1. Metabolism of ethanol in the hepatocyte and schematic representation of its link to fatty liver, hyperlipemia, hyperuricemia, hyperlactacidemia, ketosis and hypoglycemia. Pathways which are decreased by ethanol are represented by dashed lines. ADH: Alcohol dehydrogenase, MEOS: Microsomal ethanol oxidizing system.

quences of the shift in NADH/NAD have been pursued, including the inhibition of citric acid cycle activity to be discussed subsequently.

Major progress in the study of the hepatic and metabolic effects of ethanol was made by the demonstration that in addition to the changes in intermediary metabolism directly linked to the oxidation of ethanol through the shift in NADH/NAD ratio, chronic ethanol ingestion results in more lasting changes in both the function and structure of various hepatic subcellular organelles. That alcoholic liver injury is associated with striking alterations in various hepatic organelles was known (Lieber, 1966), but it was not clear whether this was due to ethanol itself, the associated nutritional deficiencies, or some other factors. This field was opened for study by the development of a new experimental model for alcoholic liver injury in the rat by incorporation of ethanol in liquid diets (Lieber, Jones, Mendelson and DeCarli, 1963; Lieber, Jones and DeCarli, 1965; DeCarli and Lieber, 1967). Whereas by conventional feeding procedures (addition of ethanol to drinking water), ethanol consumption is insufficient to result in liver damage (if an adequate diet is supplied), with this new technique, ethanol intake was on the average doubled. This resulted not only in fat accumulation despite the adequacy of the diet (Lieber, *et al.*, 1963, 1965; DeCarli and Lieber, 1967) but in addition, marked ultrastructural changes were noted both in the endoplasmic reticulum and the mitochondria (Iseri, Gottlieb and Lieber, 1964; Iseri, Lieber and Gottlieb, 1966). These

findings, soon confirmed in man (Lane and Lieber, 1966; Rubin and Lieber, 1967) led to a number of studies to define both the morphology and the functional significance of these striking and persistent changes produced in the liver by the chronic ingestion of ethanol. The same technique of incorporation of ethanol in a totally liquid diet, when applied to the baboon (Lieber and DeCarli, 1974), has allowed us to reproduce experimentally in the primate the full spectrum of liver injury observed in man, that is fatty liver, hepatitis and cirrhosis, establishing thereby the direct etiologic role of ethanol, independent of nutritional deficiencies (Rubin and Lieber, 1973; 1974).

EFFECTS OF ETHANOL ON THE STRUCTURE AND FUNCTIONS OF THE ENDOPLASMIC RETICULUM

Morphologic Changes and Alterations in Drug Metabolism

The first indication that ethanol may interact with the hepatic endoplasmic reticulum originated ten years ago with the observation that ethanol feeding results in a proliferation of the smooth membranes of the hepatic endoplasmic reticulum (Iseri, *et al.*, 1964; 1966). This microscopic finding was confirmed by some (Lane and Lieber, 1966; Rubin, Hutterer and Lieber, 1968; Lieber and Rubin, 1968; Rubin and Lieber, 1968a; Carulli, Manenti, Gallo and Salvioli, 1971; Pieper, Skeen, McClure and Bourne, 1972), but disputed by others (Dobbins, Rollins, Brooks and Fallon, 1972). More recently, however, the proliferation of hepatic smooth endoplasmic reticulum has been established on a firm basis by the demonstration of an increase in both phospholipids and total protein content of the smooth membranes (Ishii, Joly and Lieber, 1973). This increase in the mass of the smooth endoplasmic reticulum was associated with an enhancement of some of the key components of the microsomes, such as cytochrome P-450. Again this increase predominated in the smooth fraction and it exceeded the rise in total protein, thereby resulting in an increase in the content of cytochrome P-450, even when expressed per mg of microsomal protein (Ishii, *et al.*, 1973). Similar changes after alcohol feeding were observed in the activities of a variety of microsomal drug detoxifying enzymes, both in rats (Rubin, *et al.*, 1968; Rubin, Bacchin, Gang and Lieber, 1970a; Joly, Ishii, Teschke, Hasumura and Lieber, 1973) and in man (Rubin and Lieber, 1968b), an observation soon confirmed by other groups (Ariyoshi, Takabatake and Remmer, 1970; Singlevich and Barboriak, 1971). These effects were associated with an accelerated blood clearance of drugs, including ethanol (Misra, Lefèvre, Ishii, Rubin and Lieber, 1971). The enhanced drug metabolism could readily be explained on the basis of the increased activity of the microsomal drug detoxifying enzymes. This raised the question whether a similar mechanism may be implicated with ethanol. This possibility was supported by the observation that contrasting with the pretreatment with ethanol (which enhances the activity of drug metabolizing enzymes) the presence of ethanol inhibits it (Rubin and Lieber, 1968b), sometimes in a competitive manner (Rubin, Gang, Misra and Lieber, 1970b). Other groups described comparable inhibition of drug metabolism by ethanol (Schüppel, 1971; Cohen and Mannering, 1973). The possibility that the liver microsomes may be involved in ethanol metabolism was strengthened by the report of Orme-Johnson and Ziegler (1965) that the microsomal fraction of the liver was capable of oxidizing methanol, and to a small extent also ethanol. However, the rate of ethanol metabolism was so low that this pathway seemed to account for only a negligible fraction of ethanol metabolism *in vivo*.

Subsequently, a microsomal system was described (Lieber and DeCarli, 1968; 1970a) which oxidizes ethanol at a rate ten times higher than the one of Orme-Johnson and Ziegler (1965). Moreover, this system was found to have the interesting property of adaptively increasing in activity after chronic ethanol consumption (Lieber and DeCarli, 1968; 1970a), an observation confirmed by others (Tobon and Mezey, 1971; Carter and Isselbacher, 1971). The modified type II "binding" spectrum displayed by microsomes upon addition of ethanol was also found to increase in magnitude after chronic ethanol feeding (Rubin, Lieber, Alvares, Levin and Kuntzman, 1971). The present report will emphasize some of the properties of this microsomal ethanol oxidizing system (MEOS) and its differentiation from the two other known alcohol oxidizing systems, namely the pathways involving alcohol dehydrogenase (ADH) and the peroxidatic activity of catalase (Figure 2). Changes in various other microsomal functions (such as increased lipoprotein production) will also be discussed.

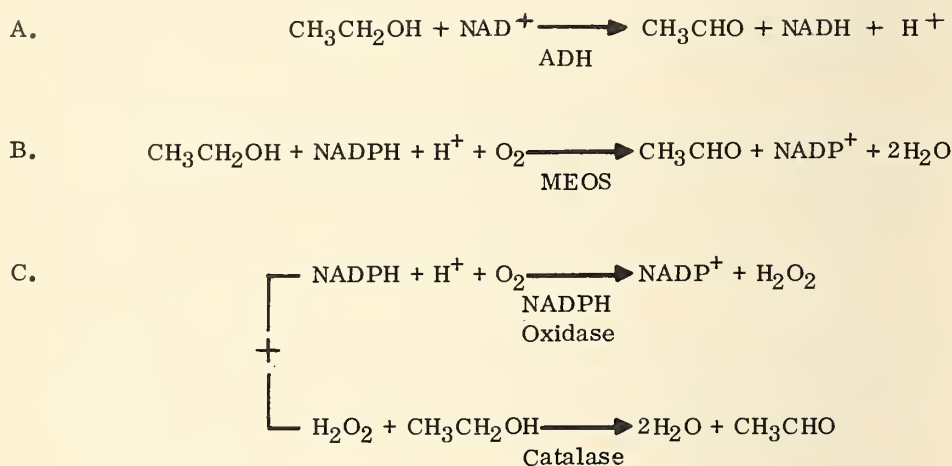


Figure 2. Hepatic ethanol oxidation by (A) alcohol dehydrogenase (ADH), (B) microsomal ethanol oxidizing system (MEOS), (C) a combination of NADPH oxidase and catalase.

The Microsomal Ethanol Oxidizing System (MEOS)

Characterization of MEOS. — MEOS can be readily differentiated from ADH (Lieber and DeCarli, 1968; 1970a) on the basis of its cofactor requirements (NADPH rather than NAD), its pH optimum (7 vs 10) and the effect of inhibitors; whereas ADH is extremely sensitive to the inhibitor pyrazole, MEOS is not affected, at least not by low concentrations (Lieber and DeCarli, 1970a; Lieber, Rubin, DeCarli, Misra and Gang, 1970a).

Differentiation of MEOS from catalase was the subject of a greater controversy, especially since MEOS requires NADPH which, in the microsomes, can also generate H_2O_2 upon its oxidation. H_2O_2 then could serve to promote the oxidation of ethanol by catalase (Figure 2) and the opinion has been expressed that H_2O_2 generation by NADPH oxidase is the rate limiting step for microsomal ethanol oxidation (Thurman, Ley and Scholz, 1972). By contrast, Hildebrandt and Speck (1973) found that up to 70 per cent

of the microsomal ethanol oxidation is H_2O_2 independent. Differentiation of these various ethanol oxidizing systems has been attempted on the basis of the sensitivity of catalase to inhibition by cyanide and azide. Some found MEOS to be less affected by these inhibitors than catalase (Lieber and DeCarli, 1970a; Lieber, Rubin and DeCarli, 1970b; Hildebrandt and Speck, 1973), whereas others did not (Carter and Isselbacher, 1971; Thurman, *et al.*, 1972). It has been pointed out however that the degree of inhibition is affected by the ratio of hemoprotein to the capacity of the H_2O_2 generating system (Oshino, Oshino and Chance, 1973). By varying the latter, experimental conditions were created in which for a given microsomal preparation and therefore for the same heme content, rates of ethanol oxidation were similar both for the NADPH and the H_2O_2 generating system (Figure 3). Under these conditions, inhibitory effects of azide were strikingly different in both systems (Figure 3) (Lieber and DeCarli, 1970a). If the oxidation of ethanol to acetaldehyde in the presence of a NADPH generating system were mediated by catalase, it should be inhibited to the same extent as the acetaldehyde production in the presence of a H_2O_2 generating system, but obviously this was not the case (Figure 3). Inhibitor studies however are rarely conclusive and therefore attempts were made at physical separation of the catalase from the MEOS. This was achieved by solubilization of the microsomes according to a modification of the method of Lu and Coon (1968) with elution by KCl gradients on DEAE-cellulose columns (Teschke, Hasumura, Joly, Ishii and Lieber, 1972). The purified MEOS fraction is rich in cyto-

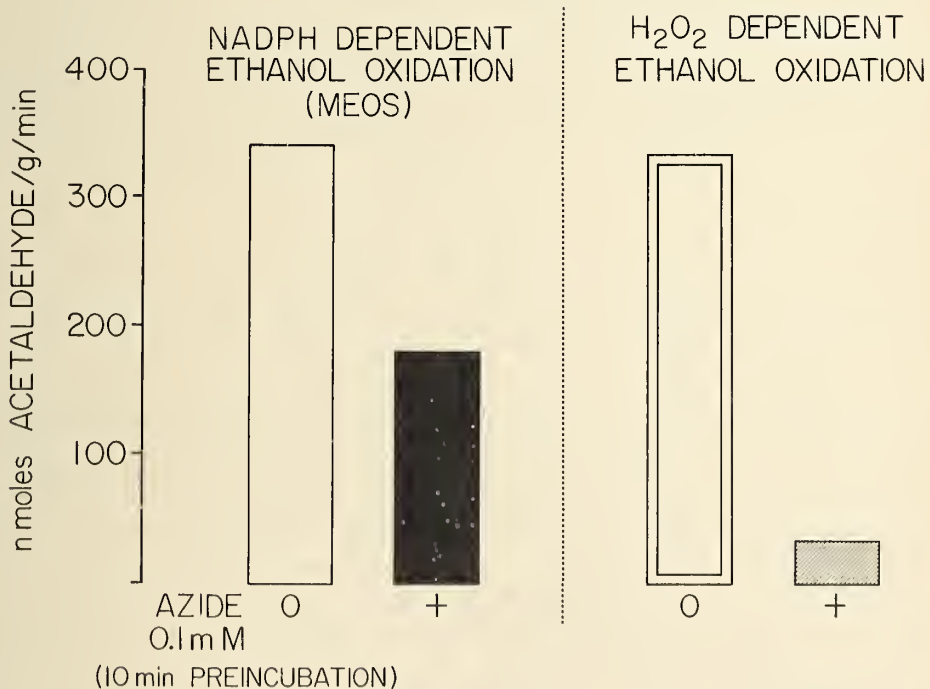


Figure 3. Effect of azide (0.1 mM) on NADPH and H_2O_2 dependent ethanol oxidation: Representative experiment carried out with the same microsomal preparation for all assays shown. The experimental conditions were as described before (Lieber and DeCarli, 1970). The results are expressed in nmoles acetaldehyde produced per minute per g of liver, without correction for microsomal losses.

chrome P-450, NADPH-cytochrome *c* reductase activity and phospholipids, all known to play a significant role in drug metabolism. The respective role of these constituents for ethanol oxidation in the purified MEOS is presently being investigated. In this study, no attempt was made to separate cytochrome P-450 in its three forms recently described (Comai and Gaylor, 1973). One of them (P-450, Form I) appears to be specifically inducible by ethanol consumption (Joly, Ishii and Lieber, 1972; Comai and Gaylor, 1973). In another study, a cytochrome P-450 rich fraction capable of ethanol oxidation was isolated from the microsomes in which catalase was considered to play no role (Mezey, Potter and Reed, 1973).

The absence of catalase in our purified MEOS preparation was verified by the lack of catalatic activity in the presence of NaBO_3 (measured by the appearance of oxygen detected with the oxygen electrode) (Goldstein, 1968) or the failure of H_2O_2 to disappear as determined by the method of Lück (1963). To rule out the possibility that the peroxidatic activity of catalase may still be present though catalatic activity may not be detectable, the effect of H_2O_2 generating systems (BaO_2 , glucose-glucose oxidase, hypoxanthine-xanthine oxidase) on the capacity of the purified fraction to oxidize ethanol was studied. The purified MEOS which actively oxidized ethanol in the presence of a NADPH generating system had no activity with the H_2O_2 generating systems unless exogenous catalase was added. Moreover, whereas ethanol oxidation with the purified MEOS and the NADPH generating system was unaffected by 0.1 mM azide or cyanide, a comparable ethanol oxidizing activity in the presence of the H_2O_2 generating system and exogenous catalase was inhibited 70 - 80% by the same inhibitor concentrations (Teschke, Hasumura and Lieber, 1974). Recently, it has been reported that microsomes of "acatalatic" mice failed to oxidize ethanol (Vatsis and Shulman, 1973). However, ethanol oxidation was measured by its disappearance, an unreliable method under the conditions used. When the experiments were repeated but acetaldehyde production was assessed, ethanol oxidation was indeed found to occur in microsomes of the same strain of "acatalatic" mice (Lieber and DeCarli, unpublished observation; Vatsis and Schulman, personal communication).

Respective roles of the various ethanol oxidizing systems. — Even when large amounts of pyrazole are used, which ought to result in complete or almost complete ADH inhibition (Deitrich, Collins and Erwin, 1971; Rydberg, Buitjten and Neri, 1972; Grunnet and Thieden, 1972), ethanol metabolism was found to persist, both *in vivo* (Lieber and DeCarli, 1972) and *in vitro* in isolated perfused liver (Papenberg, von Wartburg and Aebi, 1970), liver slices (Lieber and DeCarli, 1970a) and isolated liver cells (Grunnet, Quistorff and Thieden, 1973). The rate of this nonADH mediated oxidation varied, depending on the concentrations of ethanol used, from 20 - 25% (Lieber and DeCarli, 1970a; 1972; Papenberg, *et al.*, 1970) to half or more (Grunnet, *et al.*, 1973) of the total ethanol metabolism. Additional evidence that this pyrazole insensitive residual ethanol metabolism is not ADH mediated was derived from the fact that the cytosolic redox state was unaffected (Grunnet and Thieden, 1972). The striking increase in the nonADH fraction of ethanol metabolism with increasing ethanol concentrations (Grunnet, *et al.*, 1973) is consistent with the known K_m for ADH and MEOS: whereas the former has a K_m varying from 0.5 to 2 mM (Reynier, 1969; Makar and Mannering, 1970), the latter has a value of 8 to 9 mM (Lieber and DeCarli, 1970a). It has been estimated that the H_2O_2 dependent catalase mediated ethanol oxidation has a K_m of the same order of magnitude as that of MEOS (Thurman, *et al.*, 1972). This raises the question of the respective roles of these two systems for the nonADH mediated ethanol oxidation. An indirect answer to this question can be derived from the generally accepted

view that rates of H_2O_2 mediated peroxidation are limited by the amount of H_2O_2 generated rather than the amount of catalase itself. The total capacity of the liver to generate H_2O_2 has been estimated at 40-70 nmoles/min/g of liver (Oshino, Chance, Sies and Bücher, 1973) in the presence of physiological substrates. This is only 1/10 or even less than the estimated rates of nonADH mediated ethanol oxidation (Lieber and DeCarli, 1972). Even if the rate were increased by the unusual availability of H_2O_2 , it still would only account for a minor fraction of the nonADH mediated ethanol oxidation in control rats. This interpretation is consistent with the results of Thurman and Scholz (1973) who found that menadione, although it strikingly increased H_2O_2 generation and ethanol oxidation by microsomes *in vitro*, nevertheless failed to affect the alcohol dehydrogenase independent rates of ethanol utilization by perfused livers. By contrast, the MEOS could account for the bulk of the nonADH mediated ethanol oxidation (Lieber and DeCarli, 1972). After ethanol pretreatment, both MEOS (Lieber and DeCarli, 1968; Lieber and DeCarli, 1970a) and NADPH oxidase activity (Lieber and DeCarli, 1970b) increased. The latter may serve to generate H_2O_2 but again, in view of the very low initial rate of H_2O_2 generation in the liver, a 60 - 70% rise (Lieber and DeCarli, 1970b) could hardly account for the significant increase in ethanol metabolism observed after chronic ethanol administration, both in man (Misra, *et al.*, 1971) and in rats (Lieber and DeCarli, 1970a; Tobon and Mezey, 1971). A difference in MEOS activity between ethanol treated and control rats persisted despite catalase inhibition *in vivo* with aminotriazole or pyrazole (Lieber and DeCarli, 1970a), or *in vitro* with 0.1 mM azide (Lieber and DeCarli, unpublished observation). By contrast, calculations show that when corrected for microsomal losses during the preparative procedure, the rise in MEOS activity can account for 1/2 to 2/3 of the increase in blood ethanol clearance (Lieber and DeCarli, 1972). The unaccounted-for difference may actually result from a secondary increase in oxidation via ADH, a pathway limited by the rate of NADH reoxidation. The latter could indeed be accelerated by an increase in MEOS activity, since MEOS is associated with NADPH utilization, and the NADPH-NADP and NADH-NAD systems are linked.

Indirect evidence that MEOS activity may play a role *in vivo* can be derived from the fact that other drugs (such as barbiturates) which increase total hepatic MEOS activity (Lieber and DeCarli, 1970c) were also found to enhance rates of blood ethanol clearance (Lieber and DeCarli, 1972; Mezey and Robles, 1973). Some other studies failed to verify this effect (Khanna, Kalant and Lin, 1972). In the latter investigations, however, long acting barbiturates were used and ethanol clearance was tested in close association with barbiturate administration, at a time when blood barbiturate levels were probably elevated. Under these conditions, it was found that barbiturates interfere with blood ethanol clearance (Lieber and DeCarli, 1972). Some of the differences in the results could also be related to the modes of ethanol administration. The intraperitoneal route, used by a number of investigators, was found to result in chemical peritonitis and extensive necrosis of the liver, especially of the surface areas (Strubelt, Siegers, and Breining, 1972). In addition to the increase in MEOS activity, other mechanisms could of course contribute to the acceleration of ethanol metabolism after ethanol consumption, including mitochondrial changes. The mitochondrial mechanisms which have been postulated include enhanced shuttling of the H equivalents from the cytosol to the mitochondria after chronic ethanol feeding. We failed to find evidence in favor of this possibility (Cederbaum, Lieber, Beattie and Rubin, 1973). In general, it must be pointed out that if mitochondrial changes following chronic ethanol consumption were responsible exclusively for the acceleration of ethanol metabolism, the latter should be abolished by pyrazole treatment, but this was not the case (Lieber and DeCarli, 1970a, 1972).

Alcoholic Hyperlipemia

The effects of an acute dose of ethanol on circulating lipoproteins depend on the dose of ethanol, the species and the associated diet. *In vitro*, high ethanol concentrations may decrease hepatic lipoprotein release (Schapiro, Drummey, Shimizu and Isselbacher, 1964). More recently, when livers were perfused with ethanol in concentrations more in keeping with *in vivo* conditions, no inhibition of lipoprotein secretion was found (Gordon, 1972). Similarly, contrasting with the hyperlipemia which is associated with the administration of moderate to large amounts of ethanol in man (Lieber, *et al.*, 1963; Jones, Losowsky, Davidson and Lieber, 1963), in rats, a high dose has been reported to decrease serum triglycerides (Dajani and Kouyoumjian, 1967), very low density lipoproteins (Madsen, 1969), high density lipoproteins (Koga and Hirayama, 1968) and the incorporation of glucosamine into the carbohydrate moiety of serum lipoproteins (Mookerjea and Chow, 1969). In the rat, as shown in Figure 4, an acute dose of ethanol did not produce hyperlipemia, though it was given with a fat-containing diet, known to promote the hyperlipemic effect of ethanol, as discussed elsewhere (Baraona and Lieber,

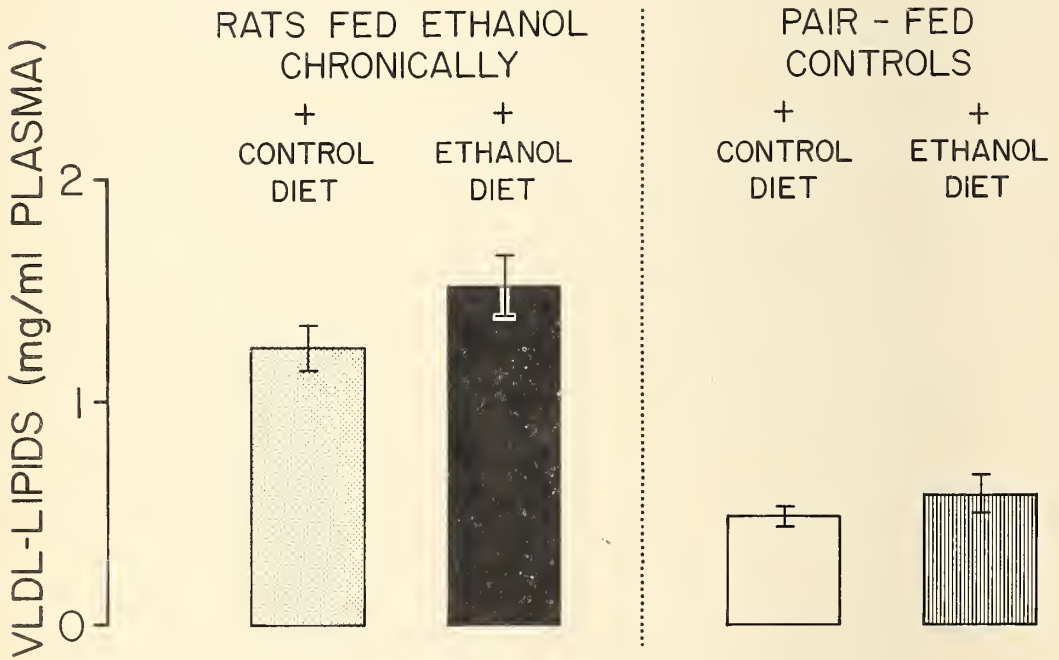


Figure 4. Comparison between acute and chronic ethanol administration on post-prandial lipemia in the rat. Animals were pair-fed liquid diets containing either ethanol (36% of total calories) or isocaloric carbohydrate (controls) for 3-4 weeks. Alcohol fed rats developed hyperlipemia in response to a load of diet with or without ethanol; by contrast, control fed rats did not develop hyperlipemia in response to an acute administration of an ethanol containing diet (3 g ethanol per kg body weight). (From Baraona and Lieber, 1973).

1973). By contrast, as shown in Figure 4, after several weeks of chronic ethanol feeding, the same dose of ethanol given with the same diet resulted in a much more striking hyperlipemia. This was associated with enhanced incorporation of labeled fatty acid into the lipid moiety of the lipoprotein and labeled amino acid into the protein moiety, suggesting enhanced lipoprotein production (Baraona and Lieber, 1970). For several experimental reasons reported elsewhere (Baraona, Pirola and Lieber, 1973), the liver is the most likely site for this enhanced lipoprotein production. Similar experiments also indicated that inhibition of peripheral utilization, though not fully ruled out, did not appear to play a major contributory role (Baraona and Lieber, 1970; Baraona, *et al.*, 1973). In man too, chronic ethanol consumption has been shown to be associated with a progressive increase in the lipemic response (Lieber, *et al.*, 1963). This alcohol effect did not result solely from caloric overload, since no comparable hyperlipemia was produced by isocaloric amounts of either carbohydrate or lipids (Losowsky, Jones, Davidson and Lieber, 1963). In man, after several weeks of alcohol intake, the hyperlipemia regressed, possibly in relation to the development of liver damage (Lieber, *et al.*, 1963). During the initial couple of weeks of ethanol administration, however, there was a progressive slow rise of circulating lipids, suggesting some type of adaptive response. Indeed, as shown in Figure 4, rats fed ethanol chronically acquire an increased hyperlipemic response even when challenged with a normal fat-containing diet devoid of ethanol. That chronic ethanol feeding may result in enzyme changes in the liver which promote lipoprotein production was then substantiated by the observation that chronic ethanol consumption produced an increase in the activity of the hepatic microsomal L- α -glycerophosphate acyltransferase (Joly, Feinman, Ishii and Lieber, 1973). It is noteworthy that fatty acids are esterified (Stein and Schapiro, 1958) and lipoproteins are formed (Jones, Ruderman and Herrera, 1967) in the endoplasmic reticulum. The mechanism whereby chronic ethanol consumption alters lipoprotein metabolism has not been clarified but it could theoretically be linked to the production of lipoproteins in the endoplasmic reticulum, especially since ethanol can be oxidized at this metabolic site and ethanol consumption results in a proliferation of the membranes of the smooth endoplasmic reticulum, as discussed before. In addition to the endoplasmic reticulum, the Golgi apparatus plays a role in lipoprotein assembly and secretion. Prominence of the hepatic Golgi apparatus was noted in man after ethanol consumption (Rubin and Lieber, 1967). More recently, in the rat this was found to be associated with enhanced activity of glycosyl transferase of the Golgi apparatus, an enzyme involved in the incorporation of the carbohydrate moiety into the lipoproteins (Gang, Lieber and Rubin, 1973).

Thus, *in vitro*, ethanol depresses hepatic lipoprotein secretion (or leaves it unaffected) and acute administration *in vivo* fails to engender significant hyperlipemia in the rat and provokes only a mild response in man; by contrast, chronic ethanol consumption produces a markedly enhanced capacity to develop hyperlipemia, both in man and in rats. This is associated with a morphologic change characterized by the hypertrophy of the subcellular hepatic organelles associated with lipoprotein production (the endoplasmic reticulum and Golgi apparatus) and is also accompanied by an increase in activity of some of the enzymes of these subcellular fractions. These changes could be viewed theoretically as an adaptive response contributing to the capacity of the liver to rid itself of the excess fat which tends to accumulate as a consequence of chronic ethanol abuse. Consistent with this interpretation is the finding that in subjects with alcoholic fatty liver, stored hepatic fatty acids appear to be the source of plasma VLDL fatty acids (Barter, Nestel and Carroll, 1972).

Energy Cost of Microsomal Oxidations

Administration of either ethanol or other drugs enhances oxygen consumption of drug pretreated animals, as compared to untreated controls (Pirola and Lieber, 1973). This could again result from the increased activity of the microsomal enzymes and the associated oxygen utilization. Furthermore, in these microsomal oxidations there is no coupling of oxidation to phosphorylation. Thus, heat is produced without conservation of chemical energy. This could conceivably be responsible for the fact that ethanol has a greater specific dynamic action in alcoholics than in normal persons (Trémolières and Carré,

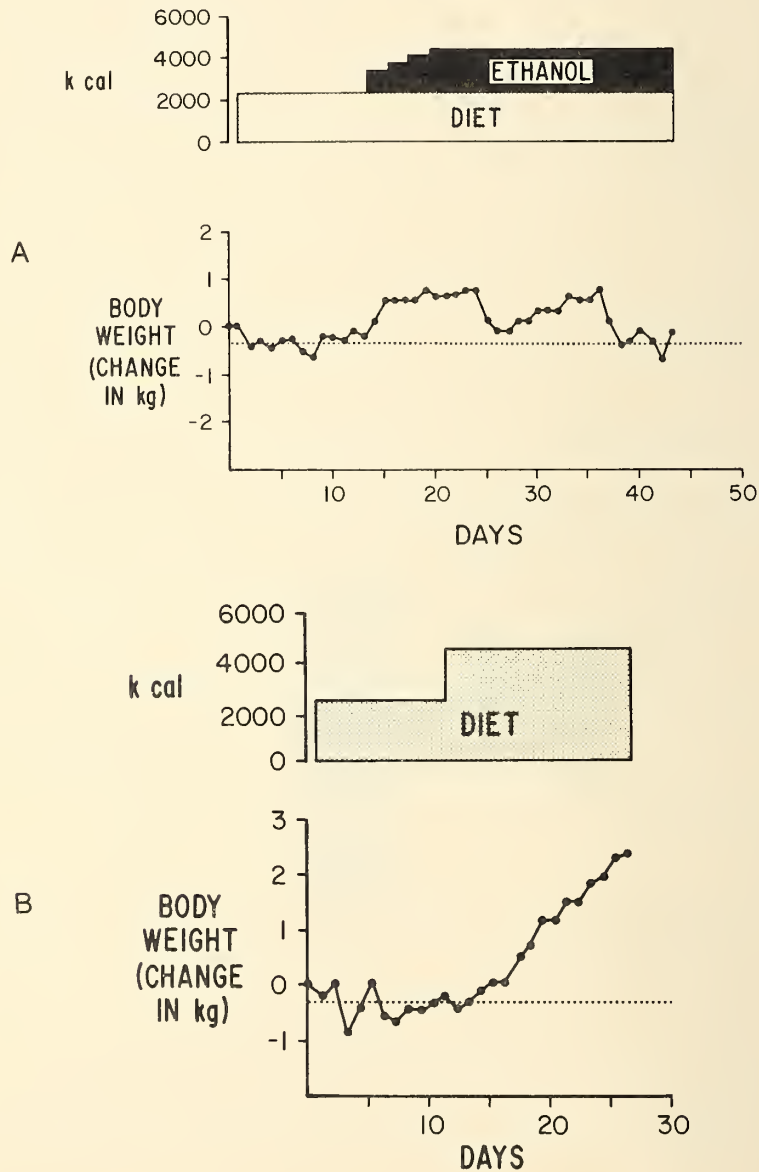


Figure 5. Effect on body weight of the addition of 2000 kcal daily as ethanol (A) or as chocolate (B) to the diet of the same subject. The dotted line represents the mean change during the control period. (From Pirola and Lieber, 1972).

1961), since microsomal ethanol oxidation might be induced in the former but not in the latter. Moreover, this effect may also cause the lesser growth (Lieber, *et al.*, 1965) in animals fed ethanol as compared to isocaloric carbohydrate, since heat produced in excess of the needs for temperature homeostasis represents energy wastage. A similar mechanism may explain, at least in part, the weight loss of subjects upon isocaloric substitution of food by ethanol and the relative lack of weight gain upon addition of ethanol to the diet compared to the effect of addition of other calories (Figure 5). Besides the energy-wasteful pathway of ethanol metabolism, there are, of course, a number of mechanisms whereby ethanol might affect the efficient disposal of ingested calories, such as interference with digestion and absorption (Lindenbaum and Lieber, 1971), but this was not the case in the study shown in Figure 5. Ethanol might also enhance other catabolic pathways that are not effectively coupled with the formation of high-energy phosphate bonds, such as the initial steps in amino acid degradation (Rodrigo, Antezana and Baraona, 1971). Moreover, acetaldehyde may uncouple oxidative phosphorylation in mitochondria (Cederbaum, Lieber and Rubin, 1974). In any event, as shown in Figure 5, concerning body weight, ethanol calories do not fully "count", at least at a relatively high ethanol intake, and especially in alcoholics.

ALCOHOLIC HEPATITIS, CIRRHOSIS AND ALTERATION OF COLLAGEN METABOLISM

It has been known for a long time that alcoholics may display liver complications of a varying degree of severity ranging from the still reversible fatty liver to the alcoholic hepatitis and finally irreversible cirrhosis. The relationship between alcoholic fatty liver and alcoholic hepatitis and cirrhosis has been the subject of much debate. It is usually accepted that cirrhosis (characterized by extensive scarring or fibrosis) may be, at least in part, a consequence of the necrosis and inflammation associated with the alcoholic hepatitis. Whether the fatty liver is a precursor for the hepatitis has been less well accepted. It must be pointed out however that although hepatic fat accumulation by itself may be harmless, it reflects a severe metabolic disturbance in the liver. It is possible that this disturbance, when exaggerated, may eventually engender irreversible damage of the hepatocyte. Necrosis in turn could lead to inflammation, resulting in "alcoholic hepatitis". Indeed, comparable electron microscopic changes of the mitochondria accompany alcoholic hepatitis (Svoboda and Manning, 1964) and the fatty liver produced experimentally by the administration of alcohol (Iseri, *et al.*, 1966; Lane and Lieber, 1966; Rubin and Lieber, 1967; Lieber and Rubin, 1968). The mass of rough endoplasmic reticulum measured chemically has also been found to be decreased (Ishii, *et al.*, 1973), corresponding to the reduction in rough endoplasmic reticulum seen by electron microscopy after ethanol consumption (Lane and Lieber, 1966; Iseri, *et al.*, 1966; Rubin and Lieber, 1967). Alteration of the rough endoplasmic reticulum was also found in patients with alcoholic hepatitis (Svoboda and Manning, 1964). Although the alcoholic fatty liver is not an inflammatory condition, and is distinguishable from alcoholic hepatitis by light microscopy, the remarkable similarity of the ultrastructural features in the hepatocytes suggests that the former may represent the precursor of the latter. Indeed, in baboons fed ethanol as part of a nutritionally adequate liquid diet (Lieber and DeCarli, 1974), alcoholic hepatitis and cirrhosis developed subsequent to the steatosis (Rubin and Lieber, 1974). It is noteworthy however that experimentally, the alcoholic fatty liver is associated with a moderate increased formation and accumulation of hepatic collagen and enhanced activity

of collagen proline hydroxylase prior to the development of hepatitis (Feinman and Lieber, 1972). It is unclear to what extent these early changes in collagen metabolism also contribute to the ultimate development of cirrhosis, the pathogenesis of which is still unknown, nor is it apparent by which mechanism ethanol produces the marked lesions in the membranes of the mitochondria and endoplasmic reticulum already seen at the stage of the fatty liver. In particular, the mechanism whereby chronic ethanol consumption results in an enhanced activity of peptidyl proline hydroxylase is unknown. As pointed out before (Lieber and Davidson, 1962; Lieber, 1968, 1973), a number of metabolic effects of ethanol can be linked to the enhanced NADH/NAD ratio secondary to the metabolism of ethanol. Among other effects, the enhanced NADH/NAD ratio also reflects itself in an increased lactate-pyruvate ratio that results in hyperlactacidemia (Lieber, Leevy, Stein, Cherrick, Abelman and Davidson, 1962a; Lieber, Jones, Losowsky and Davidson, 1962b) because of both decreased utilization and enhanced production of lactate by the liver (Lieber, 1969). The hyperlactacidemia contributes to acidosis and also reduces the capacity of the kidney to excrete uric acid, leading to secondary hyperuricemia (Lieber, *et al.*, 1962b). A fascinating but as yet hypothetical consequence of the increased availability of lactate may be the stimulation of collagen production. Indeed, elevated lactate concentration is associated with enhanced activity of collagen proline hydroxylase *in vitro* (Green and Goldberg, 1964) and *in vivo* (Lindy, Pedersen, Turto and Uitto, 1971). Such a mechanism could conceivably apply to the increased activity found in alcohol fed rats and baboons (Feinman and Lieber, 1972), but this remains to be investigated. The altered redox potential, however, appears to play a key role in explaining the fat accumulation. Indeed, both decreased lipid oxidation and enhanced lipogenesis can be linked to the metabolism of ethanol and the associated increased generation of NADH. The increased NADH/NAD ratio raises the concentration of α -glycerophosphate (Nikkila and Ojala, 1963) which favors hepatic triglyceride accumulation by trapping fatty acids. In addition, excess NADH may promote lipogenesis (Lieber and Schmid, 1961) possibly by the mitochondrial elongation pathway or transhydrogenation to NADPH. Theoretically, enhanced lipogenesis can be considered a means for disposing of the excess hydrogen generated by ethanol oxidation in the liver. Some of the excess hydrogen equivalents can be transferred into the mitochondria by various "shuttle" mechanisms. These hydrogen equivalents supplant the citric acid cycle as a source of hydrogen. Ethanol can block the activity of the citric acid cycle in at least two ways, both of which are consequences of the change in the NADH/NAD ratio. Increased NADH/NAD slows those reactions of the cycle which require NAD. Indeed, a major site of interaction of ethanol on the citric acid cycle was found to be on α -ketoglutarate oxidation (Ontko, 1973). Moreover, the redox change associated with ethanol oxidation decreases hepatic concentration of oxaloacetate (Williamson, Browning and Scholz, 1969), the availability of which controls the activity of citrate synthetase. Under these conditions, the mitochondria will utilize the hydrogen equivalents from the ethanol rather than oxidize the two carbon fragments derived from the fatty acids. The liver is then confronted with the task of disposing of the accumulating fatty acids. Two mechanisms are available for that purpose, namely ketogenesis and lipoprotein production and secretion. The capacity of these mechanisms, however, is rather limited in comparison to the huge metabolic overload ethanol may represent. Indeed, when a liver is exposed to ethanol for the first time, ketogenesis is only slightly increased (Williamson, *et al.*, 1969; Lindros, 1970), unchanged (Lefèvre, Adler and Lieber, 1970) or even reduced (Ontko, 1973). Similarly, as discussed before, when rats are given ethanol for the first time, no hyperlipemia develops (Baraona, *et al.*, 1973). Although, after chronic ethanol adminis-

tration, a progressive increase of the capacity for ketogenesis (Lefèvre, *et al.*, 1970) and lipoprotein production was observed to develop, this "adaptive" response of the liver may not be sufficient to prevent the fat accumulation, which can be secondarily enhanced by the decrease in mitochondrial function (Cederbaum, Lieber, Toth, Beattie and Rubin, 1973) and by the exhaustion of the lipoprotein response (Lieber, *et al.*, 1963) which eventually develops. As discussed in this symposium (Rubin, 1974), ethanol interferes with mitochondrial protein synthesis; furthermore, acetaldehyde, a metabolite of ethanol, exerts some toxic effects upon the mitochondria, albeit at high concentrations (Cederbaum *et al.*, 1974). It will be the task of future studies to determine to what extent these as well as other possible mechanisms contribute to the conversion of the fatty liver, a still reversible lesion, to the irreversible stages of the disease. It is obvious that there is a limit to the capacity of the liver to cope with the metabolic overload that ethanol represents. The magnitude of the load is not always appreciated. It is noteworthy indeed that the human species sometimes indulges in extraordinarily large amounts of ethanol, occasionally in excess of the caloric contribution of all other nutrients combined. When we ingest such large amounts of alcohol, the body is presented with a major problem of disposal: both the kidney and lungs are very inefficient in excreting alcohol. Furthermore, unlike other nutrients (such as lipids or carbohydrates), alcohol cannot be stored in the body. The only effective way to rid the body of the ethanol is oxidation and the only organ which contains a significant amount of the enzymes needed is the liver. The rate of disappearance of ethanol from the blood is indeed remarkably decreased or halted by hepatectomy or procedures damaging the liver (Thompson, 1956). Moreover, the predominant role of the liver for ethanol metabolism was shown directly in individuals with portacaval shunts undergoing hepatic vein catheterization (Winkler, Lundquist and Tygstrup, 1969). Extrahepatic metabolism of ethanol, although it occurs, is small (Larsen, 1959; Forsander, Raiha and Suomalainen, 1960). In the last analysis, it is probably this relative organ specificity of alcohol, coupled with the lack of an effective feedback control mechanism for regulation of rates of ethanol metabolism in the liver, which underlies the hepatotoxicity of ethanol. In any event, the reproduction, in the baboon fed ethanol as part of a nutritionally adequate liquid diet (Lieber and DeCarli, 1974), of the entire spectrum of liver lesions observed in the alcoholic (fatty liver, hepatitis and cirrhosis) (Rubin and Lieber, 1974), now offers us an experimental model to study the pathogenesis of alcoholic liver injury.

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On the Etiology of Alcoholic Liver Cirrhosis

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The association of both fatty livers and cirrhosis with the chronic consumption of alcoholic beverages is too well known to need documentation. But for most of this century there have been many who have postulated that the etiologic relationship between ethanol and hepatic damage is not that of a simple and direct cause-and-effect as is more generally accepted for hepatotoxins such as carbon tetrachloride. To the contrary, evidence has accumulated from epidemiologic studies in man and from experiments with animals, which makes it probable that much, if not all, of the effect of the chronic consumption of large amounts of alcohol on the liver is brought about indirectly because it is an unusually rich source of nutritionally 'empty' calories (over 7 cal. per ml). According to such a view the addition of a thousand or even more of alcohol-derived calories to a diet, nutritionally adequate by itself, may induce important deficiencies relative to the total daily energy intake (diet plus alcohol). Thus the liver may be seriously damaged by alcohol-induced inadequacies of protein and essential food factors. In accord with the assigned title, consideration will be given here only to studies concerned with alcohol and cirrhosis (or at least hepatofibrosis) and little mention will be made of the many papers concerned only with the development of a fatty liver. It is true that in man the cirrhosis associated with alcohol is preceded by a fatty liver, but it has also been established, at least in animals, that there are many types of fatty liver which do not progress into cirrhosis. Therefore, to avoid confusion on this basis, only those investigations dealing with alcohol and cirrhosis, as distinct from fatty liver alone, will be included here.

Amongst large groups of alcoholics segregated in sanatoria for the purpose of treating the addictive or socioeconomic consequences of their drinking habits, in only a small minority (1 in 12 in one study; Sherlock, 1968) can evidence of cirrhosis be detected.

Therefore in the majority of such alcoholics, some factor or factors must be operating to give them hepatic immunity from any directly harmful effect of the alcohol on their livers. Although genetic background may be important in this connection, variations in diet accompanying the alcohol ingestion can be reasonably suspected to account for the great variation in the livers.

The data to be presented have all been previously published. The objective is to bring together the evidence supporting the view that the effect of alcohol on the liver can be importantly modified by manipulation of the diet in experimental animals. Less complete evidence supports the extrapolation of these conclusions to man. Two pertinent questions are relevant to the problem.

Can cirrhosis be produced in animals by alcohol? For many years, all attempts failed. F. B. Mallory devoted much of his life to this objective and quite without success. And still to this day, no one has reported the development of cirrhosis in an experimental animal which has been allowed to consume a diet normal in amount and quality, along with the alcohol. This failure has been particularly prominent whenever a so-called "stock" or "normal laboratory ration" ("chow," etc.) has been employed. The importance of these negative results should not be overlooked simply because they were "negative". In toto these failures, many of which of course were never published, represent a formidable body of evidence that the consumption of even large amounts of alcohol over long periods by experimental animals given normal diets will not produce significant degrees of either loss of hepatic function or distortion of normal structure. Given these limitations, in animals alcohol does not produce alcoholic cirrhosis. Based on these findings and others, it was therefore possible in 1934 for Moon to state: ". . . it must be concluded that experimental evidence has not substantiated the belief that alcohol is a direct cause of fatty liver" (let alone cirrhosis). More than three decades later Porta and Gomez-Dumm (1968) in developing their method of persuading rats to drink large amounts of dilute alcohol by sweetening the solution with sucrose, found again that when the diet offered to the animals consisted of laboratory chow little or no change could be observed in the livers.

In man, the cirrhosis associated with consumption of alcohol is preceded by a fatty liver. It was reasonable to assume that such might be the case in the experimental animal. A large number of experiments, most of relatively short duration, did demonstrate that administration of alcohol to animals would, under precisely defined and limited dietary conditions, result in abnormal accumulation of hepatic fat and even cirrhosis. These experiments are well known and will be mentioned only briefly.

1. In dogs, Connor and Chaikoff (1938) found that by feeding them a low-protein, high-fat diet accompanied by large amounts of alcohol by stomach tube, fatty and even cirrhotic livers resulted. Later it was postulated that the combination of the alcohol with a high-fat, low-protein diet had induced a relative lipotropic deficiency. This view was strengthened by the experiments of Lowry and co-workers (1942) who produced fatty livers in choline-deficient rats fed alcohol, and a degree of cirrhosis in some. It should be noted that the work of Connor and Chaikoff, thirty-five years ago probably is the first recorded production of cirrhosis in experimental animals given alcohol.

2. The basal diets accompanying the alcohol in the above experiments, in themselves lead to hepatic abnormalities which were intensified by the addition of calories from alcohol. Therefore it was difficult to conclude that the alcohol had in fact caused the cirrhosis. To clarify this point, Best and co-workers (1949) extended these studies by devising a diet, which when consumed alone (without alcohol) by rats completely protected their livers. This diet however contained no excess of lipotropic factors (choline,

methionine, methionine-containing protein) so that when calories from either alcohol or carbohydrate were superimposed, fatty livers were produced. But additional choline along with the added alcohol prevented the fatty livers. In these experiments, some livers of rats consuming alcohol and the basal complete diet without extra lipotropic factors, in addition to fatty change developed hepatofibrosis but not what was judged to be true cirrhosis. The amount of alcohol (15% in drinking water v/v) was probably insufficient and the duration of the experiments was probably too short for the production of cirrhosis as judged by knowledge derived from subsequent studies. In any event, it was abundantly clear from these experiments that the diet accompanying the intake of alcohol was of great importance in determining the fate of the animals' livers. Studies at Yale (Klatskin, Gewin and Krehl, 1951) essentially confirmed these conclusions.

3. The introduction of an all liquid diet into alcohol-experiments (Lieber, Jones, Mendelson and DeCarli, 1963) rekindled interest in the animal models because it enabled studies to be carried out in which the animals consumed appreciably larger amounts of alcohol (36% of total calories) than hitherto; (in the Toronto studies above, rats would consume no more than some 28% of total calories as alcohol). Drs. Lieber and Rubin in New York followed up this initial report with an impressive series which continues to this day exploring the inter-relationships between alcohol and hepatic injury. The next advance was the development of the Porta model utilizing sweetened alcoholic solutions in place of drinking water (Porta and Gomez-Dumm, 1968). They found that the rats would consume even greater percentages of their total calories in the form of alcohol: up to 46% or even more. Up to this period, the stream of papers dealing with alcohol and the liver from a number of centers including that at Mount Sinai and our own at Toronto were chiefly concerned with attempts to unravel the complexities of diet, liver and the abnormal accumulation of fat. So from 1951 until 1969, reports of attempts to reproduce alcoholic cirrhosis in animals did not appear. But in 1969, with the realization that rats could be persuaded to take between 40 - 50% of their total calories in the form of alcohol by using the Porta model, it seemed that it now might be possible to actually produce alcoholic cirrhosis and such did prove the case (Porta, Koch and Hartroft, 1969).

Rats were given a semisynthetic basal diet (lipotropic value¹ 29) which consumed by itself would produce no more than a slight to mild fatty liver, but which when diluted by the sweetened alcohol in the Porta drinking fluid provided a final caloric proportion of 5% derived from protein, 16% from fat, 35% from carbohydrate and 46% from alcohol. The dilution by the "empty" alcohol-derived calories now reduced the lipotropic value to only 12. Three control groups were provided, whereby in one the alcohol was replaced isocalorically by sucrose, in another by fat and in the third control group the alcohol was replaced by an ideal mixture of protein, fat and carbohydrate giving a well-balanced total food mixture with a more than adequate lipotropic value (greater than 40 under most of our experimental conditions will prevent fatty livers in rats). Animals in the first three groups developed cirrhosis, whether the dilution of the basal diet and of its protein and choline contents had been accomplished by the addition of "empty" calories derived from alcohol, carbohydrate or fat. The cirrhosis grossly exhibited the characteristics of a multilobular phase at the end of six months. Microscopically the picture was that of a classical fatty multilobular cirrhosis exhibiting the features familiar to all in alcoholic cirrhosis of man. By electron microscopy mitochondrial enlargement was striking as was the dilation of the smooth ergastoplasmic reticulum. These ultrastructural hepatocytic

¹The lipotropic value or index represents the content of choline or its dietary precursor methionine expressed as milligrams per 100 calories of diet. Methionine is considered to have one-third of the lipotropic activity of choline, per milligram, because the former has only one labile methyl group compared with choline's three.

changes have been prominent in livers of rats of all our experiments concerning alcohol reported to date. I should emphasize that the change in the ER represents only vacuolation and not proliferation as observed by Rubin and Lieber (1969). In our studies of livers of rats involving both the acute and chronic models of alcohol consumption, measurements of ergastoplasm by the method of Loud (1968) have not indicated any increase in membranes of smooth ER (Porta, Sugioka and Hartroft, 1969).

The livers of the final control group of rats fed the basal diet supplemented with a well balanced mixture of fat, protein and carbohydrate to provide the same number of calories as did the alcohol, fat or carbohydrate supplements of the first three groups, were completely normal by all parameters.

With additional groups to the above three it was shown that the cirrhotic effect of the alcohol as seen in the "lead" group, could be prevented by suitable dietary modification. Adequate levels of protein without a choline supplement prevented cirrhosis. High supplements of choline, without raising the low-protein level, completely protected the livers of rats consuming the Porta drinking fluid although growth rates were low because of the inadequate dietary protein. The addition of ten times the normal level of vitamin mixture (without choline) prevented cirrhosis although fatty changes and mitochondrial enlargement were present. In later experiments it was found that folic acid and vitamin B₁₂ were probably responsible ingredients in the complete mixture (Porta, Hartroft and Koch, 1971).

Therefore, in answer to the question posed at the beginning of this section, in rats it is possible to convert a noncirrhotic basal diet to a cirrhosis-producing regimen by the addition of alcohol in sufficient amount (40 - 50% of total calories) that the percentage of protein, choline, vitamin B₁₂ and folic acid are thereby lowered below critical values. In this context it can be stated that cirrhosis has been produced in animals by giving them alcohol to drink. But providing equal numbers of calories from either fat or carbohydrate will have a comparable cirrhotic effect. In any case, appropriate modification of the basal diet to provide adequate protein and lipotropic factors in proportion to the additional calories will prevent the cirrhotic action of the alcohol.

We are well aware that extrapolation of these conclusions from rat to man may to some appear speculative. Indeed, investigations in man by the group at Mount Sinai Medical School and dealt with by them elsewhere in this symposium (Lieber, this book) would postulate that alcohol may have a more directly toxic effect on the liver of man than that of the rat. But dietary studies of alcoholics with hepatic damage support the results obtained with the animals. "Skid-row" alcoholics in Chicago were found some years ago to consume a high carbohydrate, low-protein diet not unlike the basal diet which proved to be cirrhotic for our rats when diluted with alcohol (Figueroa, Sargent, Imperiale, Morey, Paynter, Vorhaus and Kark, 1956). Leevy (1962) in a group of patients with hepatic dysfunction ranging from mild to severe, found that the degree of liver damage correlated neither with the amount nor the duration of intake of alcohol but did correlate inversely with the quality of the diet accompanying the alcohol — the poorer the diet, the more severe the cirrhosis.

If alcohol is a hepatotoxin under the conditions it is consumed by man, some explanation must be found for the fact that so many alcoholics do not develop significant amounts of hepatic damage. Clearly there is no simple dose-response phenomenon for alcohol in the case of man as there is for the proven hepatotoxins such as carbon tetrachloride. At any rate there is now abundant evidence that whatever direct toxic effect alcohol may have or may not have for the human liver, its action can be vastly modified by the accompanying diet. Certainly alcohol is the only suspected hepatotoxin,

administration of which imposes a major caloric burden on the liver. In this regard it is unique.

In the above experiments, the livers of the animals were all normal at the time the alcohol-regimens were instituted. But very possibly the effect of alcohol on an already damaged liver could be quite different. It could be argued that although suitable provision of dietary factors can protect a normal liver from a potential hepatotoxic effect of alcohol, such would not succeed if cirrhosis was already established. This speculation prompted the next question to be considered here.

Can a sufficiently excellent diet protect an already damaged liver from further injury by the consumption of significant amounts of alcohol? Is it possible that the consumption of such a diet will even enable the pre-existing damage to regress despite the simultaneous consumption of alcohol? The answer to both questions is in the affirmative as shown by two differently designed experiments involving rats (Takada, Porta and Hartroft, 1967; Porta, Koch and Hartroft, 1972) and by a study in alcoholic man (Reynold, Redeker and Kuzma, 1965). The same problem has been studied in a somewhat different manner by Takeuchi and Takada in Japan and their results will be presented later.

In the first of the two animal studies, the experiment was divided into two phases. Phase 1 of 8 months duration consisted of feeding all animals a low-choline, low-protein diet which resulted in degrees of cirrhosis, confirmed at laparotomy, comparable on gross examination to that shown previously. At the end of phase 1, all rats were distributed among three groups — the first (A) served as an untreated, negative control and were continued on the basal cirrhogenic diet for the next three months during which time in one-third of them, their cirrhosis progressed to a fatal outcome. The other two groups (B and C) were transferred for the same three months to either a liquid diet providing 23% of total calories as protein, 15% as carbohydrate, 26% as fat and 36% as alcohol; or to a comparable but nonalcoholic liquid diet in which 24% of calories were supplied by carbohydrate, 40% by fat and 36% by protein. The lipotropic values of these diets were high — 81 and 115 respectively.

Regardless as to whether or not the therapeutic diets (B,C) contained alcohol, as soon as the animals entered phase 2 they improved in every parameter examined including growth in body weight, return towards normal levels of previously elevated serum glutamic-oxalacetic transaminase activity, serum alkaline-phosphatase activity, and previously abnormal ratio of albumin to globulin reverted to the normal. When killed, the livers of Group A (the negative control group) were of course severely cirrhotic whereas those of both the treated groups, although not normal, were greatly improved whether or not they had consumed alcohol during phase 2 while receiving the superdiet. Histologic examination and measurement of total collagen, (and of soluble and insoluble fractions), DNA, RNA and triglycerides all indicated a significant and even dramatic return to normal. By electron microscopy the only abnormality found in the treated groups was moderate enlargement of some of the hepatocellular mitochondria, in the group which received the alcohol (B) but all other cytoplasmic components were normal.

In the second animal experiment, two modifications from the design of the first were made. The cirrhosis was produced in 7 months with alcohol, using the Porta model as previously described (40% alcohol and 6% protein) and the therapeutic regimens during the second phase were also accomplished with the Porta model, the alcohol and the solid food being offered separately. The second stage of the experiment (treatment period) lasted 4 months. One group received 40% of its calories in the form of alcohol, but the solid food mixture was now a superdiet providing superabundant amounts of protein,

choline and vitamins. The second control group received the same superdiet under isocaloric conditions but the caloric loading was accomplished for this group by additional fat or carbohydrate instead of alcohol. Again all tests of hepatic function, abnormal at the beginning of phase 2, soon reverted to normal under the influence of the superdiet whether accompanied by 40% of total calories in the form of alcohol, or in the form of fat or carbohydrate. When killed at the end of the four months of phase 2, by both gross appearances and by light and electron microscopy, the livers had reverted to an astonishing degree toward the normal pattern. As in the first experiment, the only ultrastructural abnormality that persisted in hepatocytes of previously cirrhotic rats treated with alcohol and the superdiet was a moderate degree of enlargement of mitochondria.

In the human experiment reported by Reynolds and his colleagues (1965) even before we had carried out our comparable animal studies, a group of cirrhotics were treated with an excellent hospital diet (high in protein and choline) and were given sufficient alcohol in fruit juice to maintain significantly high levels of blood alcohol. (As the patients' livers improved the amounts of alcohol to achieve these levels had to be increased, reaching several hundred ml daily.) The control group of cirrhotics were treated with the same diet and given fruit juice alone. In both groups, whether or not given alcohol, hepatic function tests after a year were within normal range in nearly all cases, and structure as evaluated in liver biopsies had improved to a remarkable degree.

Therefore the answer to the question posed at the beginning of this second section is that in both cirrhotic animals and men under appropriate conditions, consumption of significant amounts of alcohol accompanied by therapeutic superdiets will not impede the improvement in hepatic structure and function induced by the high quality diet. Again the effect of protective dietary factors on the hepatic outcome of the consumption of alcohol was abundantly demonstrated.

CONCLUSIONS

Our assigned title was on the etiology of alcoholic cirrhosis and we have concentrated on this aspect to the neglect of the many studies concerned with the fatty stage only. For the same reason, the problem of the effect on the liver of single, large intoxicating doses of ethanol has not been considered. In any event we consider this acute model to present quite different questions than the chronic one.

From our studies reviewed here and many others, it would appear that whether or not the consumption of alcohol by experimental animals will cause cirrhosis depends on at least three conditions. They are: duration of the consumption of the alcohol, the percentage of total calories provided by alcohol, and the composition of the accompanying diet.

In none of our attempts to produce cirrhosis with alcohol, have we seen the appearance of grossly evident cirrhotic change, even of the monolobular type, within less than five months. For the production of unequivocal multilobular cirrhosis 7 to 8 months is necessary for rats in our hands. Although fatty liver and degrees of hepatofibrosis may develop within less time, it appears that at least half a year or more is required for cirrhogenesis.

The amount of alcohol is obviously of equal importance, and in discussing this factor it is necessary to consider the third one — the quality of the accompanying diet. Taking all our experiments together, including one titration type, we have found that if the alcohol supplies less than 30% of the total calories, a normally adequate diet will

completely protect the liver. It will be recalled that, in order to produce fatty liver and hepatofibrosis with alcohol providing only some 28% of total calories as in the original Toronto experiments of Best *et al.* (1949), it was necessary to devise a basal diet that contained only marginally protective levels of choline and protein when the food mixture was consumed alone without alcohol. Had a standard laboratory diet been employed, no changes would have been observed in the livers of animals consuming only this much alcohol.

But when a diet considered adequate by usual standards is diluted by more than 30% of the total calories from added alcohol, fatty change and obvious mitochondrial alterations will develop because this amount of alcohol will usually render, by caloric dilution, the otherwise adequate diet, inadequate in choline, protein and other factors. In such cases the lipotropic value will usually be found on calculation to be less than 30.

Even when the percentage calories supplied by alcohol is 36% or more, up to 45%, cirrhosis will not develop if the accompanying diet is considered to be an ordinarily adequate one. To produce cirrhosis under these conditions, it is necessary that as in the original Best experiment, the basal diet be only marginally adequate in the levels of protein, methionine, choline, vitamin B₁₂ and folic acid. When all these conditions are fulfilled, the investigator will be rewarded after 6 to 8 months by the development of an advanced degree of multilobular cirrhosis in the majority of his rats. Supplementation by additional vitamin B₁₂ or folic acid will prevent the development of cirrhosis, although permitting the accumulation of considerable excess fat and the presence of mitochondrial alterations to a mild degree. To this extent these two factors, (vitamin B₁₂ and folic acid) are effectively anticirrhotic.

Elevation of the level of dietary protein alone (but not raising the lipotropic value by using methionine-poor sources) will again prevent cirrhosis but not the development of even quite severe degrees of fatty liver. Mitochondrial changes will not be prevented either. The mechanism whereby a methionine-poor protein prevents cirrhosis but not a fatty liver under these conditions is not understood by us at present, although at one time we thought we had a solution.

Of all the protective factors, choline is the most important *single* one at least in the case of the rat. Addition of sufficient choline even when percentages of total calories provided by alcohol approach 50% and even when the diet is quite inadequate in protein, vitamin B₁₂ and folic acid, will afford complete protection for the liver from the development not only of cirrhosis, but also simple fibrosis and even abnormal accumulation of any excess fat. In the presence of abundant dietary choline, the only effect on the liver in all our experiments is the development of mild to moderate degrees of mitochondrial enlargement, providing the basal diet is reasonably balanced in regard to its proportions of fat to carbohydrate.

And finally even amounts of alcohol sufficient to provide more than 40% of total calories, will not in rats inhibit the beneficial effects of a superdiet on previously damaged livers, even when the degree of damage is severe. Such superdiets plus alcohol will bring about regression of established cirrhosis and there is evidence that this may well be true in man as well as in animals.

We are well aware that the results in all other laboratories have not always conformed to the conclusions stated above. Since members of some of these groups are here in the audience, I have made no attempt to go into detail concerning controversial points. I hope that perhaps some of these areas can be clarified later in the symposium.

In conclusion I would like to quote in part from the first paper published by Professor Best and his group in 1949 and of which I was a member at the time; "...the

diet of the alcoholic is usually deficient not only in the lipotropic substances but also in other vitamins and indeed in *all* essential food factors." We believe that this statement is as well supported today, nearly a quarter of a century later, as when it was originally written despite some suggestions to the contrary. Direct proof of our thesis in the case of man will probably never be forthcoming, for to repeat in human subjects the type of experiment we conducted in the rats to produce alcoholic cirrhosis and to prevent it with dietary supplements in others, would of course be highly unethical. But we feel that sophisticated and detailed studies of the dietary practices of alcoholics who do develop cirrhosis compared with comparably severe alcoholics who do not, should be given great attention in the future. Studies of this type already cited, strongly support the crucial role of the diet in the etiology of alcoholic cirrhosis in man. In any event, it appears reasonable even now to hope that the development of cirrhosis in members of the drinking public might be considerably diminished if they could be persuaded to adopt some of the prophylactic dietary practices suggested by the available data.

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Alcohol and its Hepatotoxic Effect

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In Japan, hepatic cirrhosis was the eighth commonest cause of death for males and the eleventh for females in 1970. The death rates from the disease in the same year were 17.6 per 100,000 for males and 7.7 for females. The incidence of alcoholism in patients with cirrhosis has been reported to vary from 52 to 86 per cent with a mean of about 75 per cent in Europe and the United States (Sereny and Devenyi, 1972). Rubin, Beattie, Toth and Lieber (1972) have stated that in New York City, most of the cirrhosis is alcoholic. In our country, on the contrary, alcohol has played a much smaller role in the etiology of cirrhosis. However, alcoholic cirrhosis has been rapidly increasing in Japan. Therefore, many Japanese investigators have become interested in the role of alcohol in the pathogenesis of hepatic injury.

In this paper, we would like to discuss (1) the etiological role of alcohol in hepatic cirrhosis in Japan, (2) experimental hepatic injury due to alcohol administration, and (3) mechanism of the development of hepatic cell necrosis due to alcohol administration.

THE ETIOLOGICAL ROLE OF ALCOHOL IN HEPATIC CIRRHOSIS IN JAPAN

Patients with hepatic cirrhosis, verified by peritoneoscopy, biopsy and/or autopsy, who were admitted to the Department of Internal Medicine, Kanazawa University Hospital and its affiliated hospitals are shown in Table I, together with the incidence of chronic alcoholism among them. Considering the daily consumption of less than 80g of alcohol is "safe" for the liver (Péquignot, 1961), the possibility that the cirrhosis is of alcoholic etiology is at most about 35 per cent in male patients, virtually zero in females. The yearly change in the proportion of alcoholic cirrhosis to total cirrhosis in 84 hospitals in

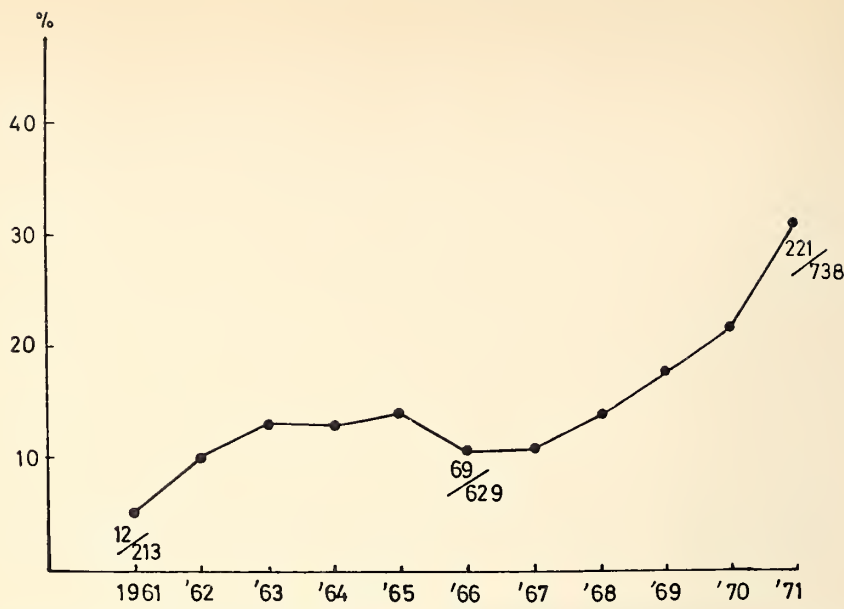


Figure 1. *Proportion of alcoholic cirrhosis to total liver cirrhosis (Data from 84 hospitals in Japan).*

Japan (Figs 1), shows that the percentage of alcoholic cirrhosis is relatively rapidly increasing. If [death rate from total cirrhosis in each year] x [percentage of alcoholic cirrhosis among total cirrhosis in the same year] is assumed to be the death rate from alcoholic cirrhosis, the death rate from alcoholic cirrhosis is steadily increasing with a significant correlation to the annual alcohol intake per one adult person (Fig. 2). The

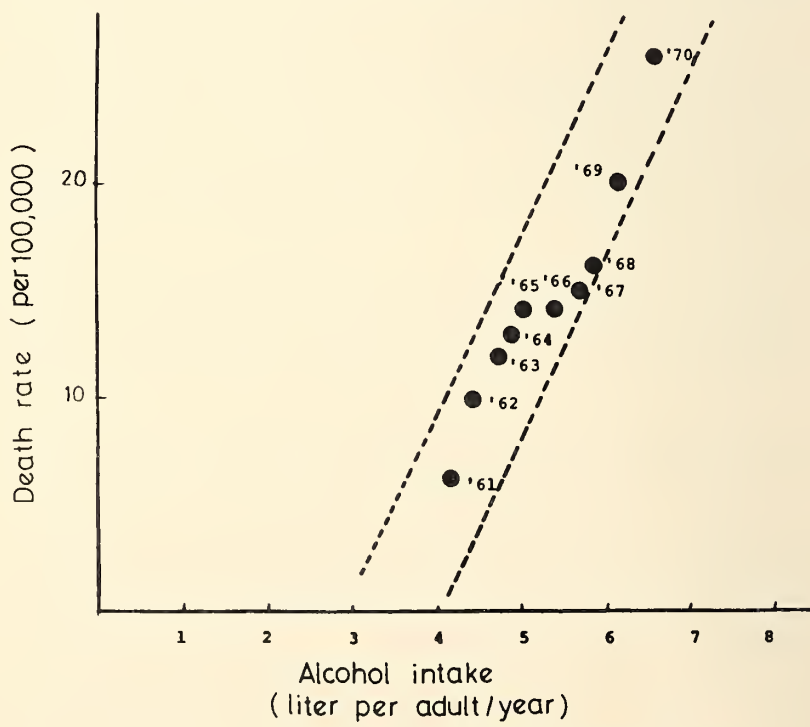


Figure 2. *Correlation between death rate from alcoholic cirrhosis and alcoholic intake in Japan.*

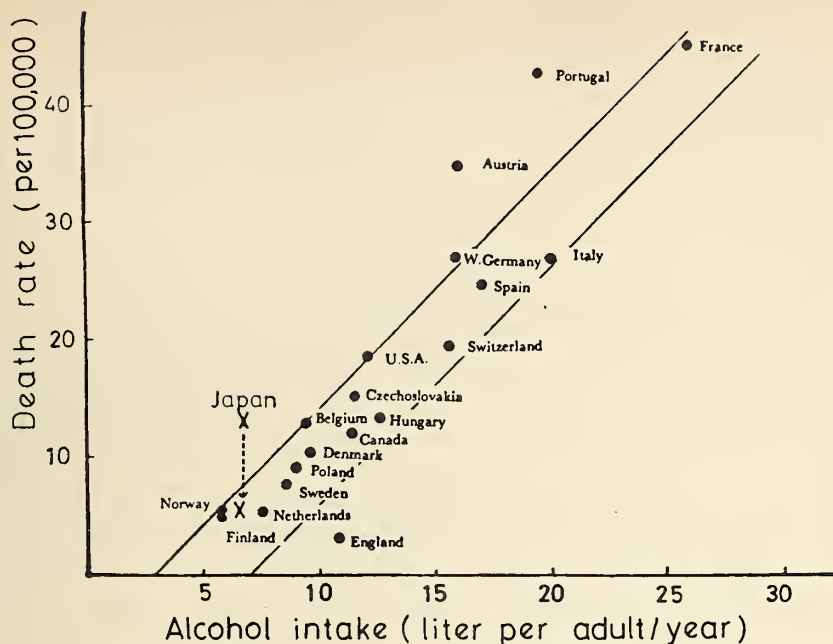


Figure 3. Correlation between death rates from cirrhosis and alcohol intake in various countries.

The figure was made by the use of data reported in de Lint, J. and Schmidt, W. *The epidemiology of alcoholism*. In Y. Israel and M. Mardones (Eds.), *Biological Bases of Alcoholism*. New York, London, Sidney and Toronto: John Wiley & Sons, Inc., 1971. Pp. 423-443.

Arrow indicates that if the percentage of alcoholic cirrhosis in Japan is regarded as 32%, i.e. the average value from 84 hospitals, the death rate from alcoholic cirrhosis moves to the new point.

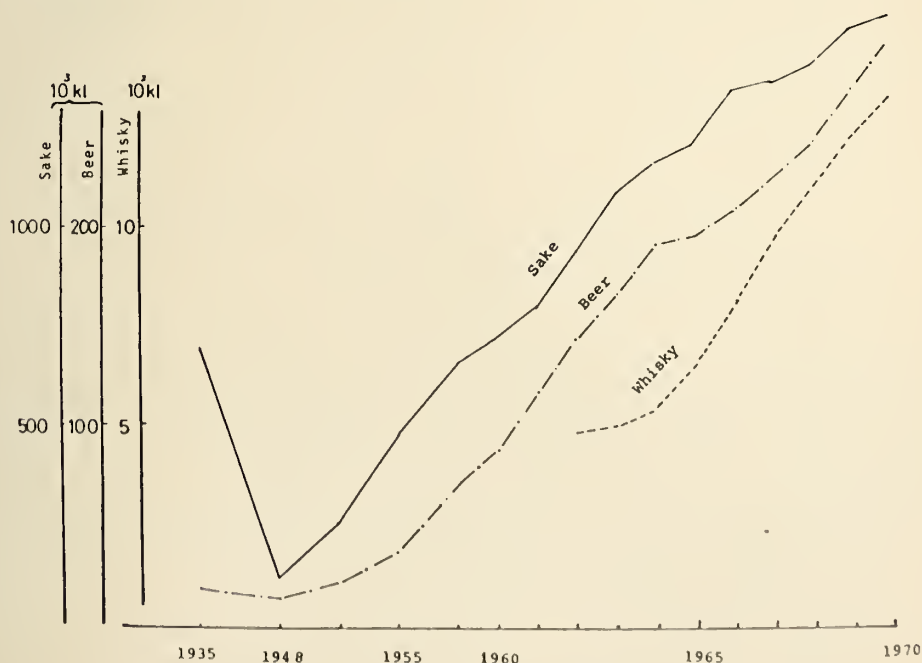


Figure 4. Yearly consumption of alcoholic beverages in Japan.

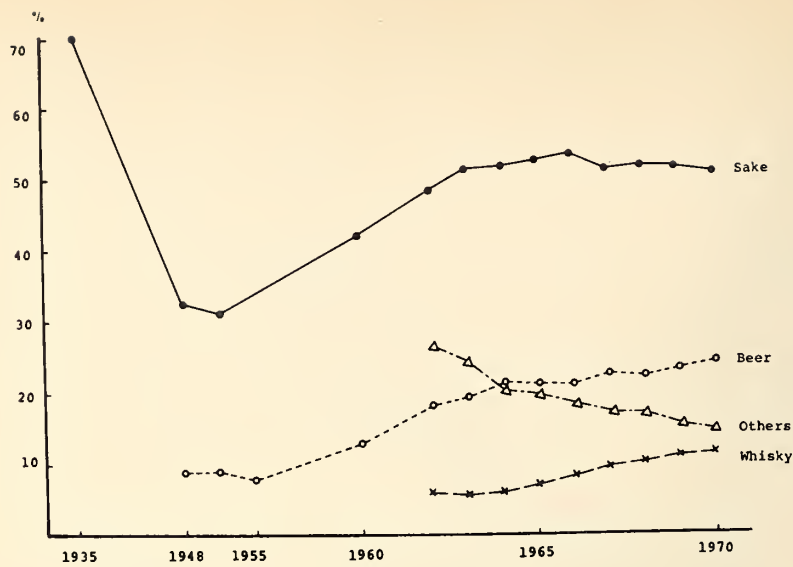


Figure 5. Percentage of alcohol intake from different beverages in Japan.

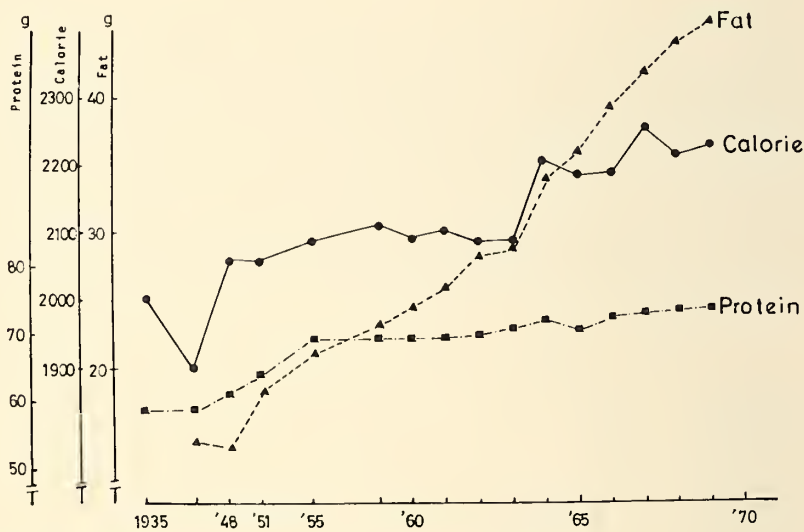


Figure 6. Yearly food consumption per capita in Japan.

correlation between the death rates from cirrhosis and the alcohol intake (per one adult person/year) in various countries in 1966 or 1967 (for Japan in 1971) indicates that the death rate in Japan is higher than that of other countries with about the same alcohol consumption (Fig. 3). However, if the percentage of alcoholic cirrhosis in Japan is regarded as 32 per cent (which is an average value of 84 hospitals in 1971), the death rate from alcoholic cirrhosis moves to the same range as other countries (arrow in Fig. 3), suggesting that the livers of Japanese people are as vulnerable to alcohol as those of European and American people. The annual consumption of alcohol in Japan has been rapidly increasing recently. In Figure 4, intakes of three main alcoholic beverages (sake, beer and whisky) are seen to be increased in proportion to each other. Since no remarkable change is noted in the percentage of each kind of alcoholic beverages after 1962 (Fig.

5), an increase in total alcohol intake itself may be responsible for the recent increase of alcoholic cirrhosis in Japan. Annual food consumption for the same period is shown in Figure 6. Since the annual consumption of protein and carbohydrate has been almost unchanged, while that of fat has progressively increased, increased fat intake might play some role in the rising incidence of alcoholic cirrhosis, in association with increase of alcohol consumption.

TABLE I

ALCOHOL CONSUMPTION IN HEPATIC CIRRHOSIS
(KUH and its affiliated hospitals, August, 1961 — July, 1973)

Alcohol consumption	Male	Female	Total
<80 g/day	151 (66.8%)	74 (98.7%)	225 (74.8%)
80-130 g/day	23 (10.2%)	1 (1.3%)	24 (8.0%)
>130 g/day	52 (23.0%)	0	52 (17.2%)
Total	226	75	301

EXPERIMENTAL HEPATIC INJURY DUE TO ALCOHOL ADMINISTRATION

There are three hepatic disorders that are thought to be related to the ingestion of alcohol: fatty infiltration of the liver, alcoholic hepatitis and hepatic cirrhosis. Fatty infiltration of the liver was long believed by many investigators to be of pathogenetic importance to the development of cirrhosis, *i.e.*, as a precursor of cirrhosis. This concept was supported by work performed mainly by the Toronto group (Best, Hartroft, Lucas and Ridout, 1949; Hartroft and Ridout, 1951; Hartroft, 1961) using experimental animals. However fatty liver in man due to excessive food intake or to endocrine causes has not progressed to cirrhosis (Galambos, 1969). Nor has fatty liver in kwashiorkor progressed to cirrhosis (Waterlow and Bras, 1957; McLaren, Bitar and Nassar, 1972). Thus, in human beings, the view that fatty liver per se leads to cirrhosis is considered to be unproven or probably erroneous (Scheig, 1970; Brunt, 1971). Furthermore, fibrosis has not developed in rats with fatty livers produced by orotic acid (Sidransky and Verney, 1965). Concerning the pathogenetic role of alcohol in hepatic injury, previous studies have placed undue emphasis on fatty liver; we believe that attention should be focused on hepatic disorders other than fatty liver, *i.e.* alcoholic hepatitis.

The histological changes in alcoholic hepatitis include hepatocytic necrosis, infiltration with polymorphonuclear leukocytes in necrotic areas and portal triads, fatty metamorphosis and alcoholic hyaline material (Mallory bodies). In contrast to steatosis, necrosis of hepatocytes and inflammation initiate fibrosis and eventually result in cirrhosis of the liver. Thus, alcoholic hepatitis is now believed to be an obligatory or potential precursor of alcoholic cirrhosis (Helman, Temko, Mye and Fallon, 1971; Insunza, Iturriaga, Ugarte and Altschiller, 1971; Popper, 1971; Galambos, 1972; Hoensch, 1972). Since the main cause of the confusion concerning the pathogenetic role of alcohol in the development of cirrhosis has been the failure to produce cirrhosis experimentally by alcohol administration, we believed that the first step in elucidating the problem would be to produce alcoholic hepatitis in experimental animals. For this purpose, we tried at first to produce hepatic cell necrosis by administration of alcohol.

Hepatic Cell Necrosis Induced by a Single Intoxicating Dose of Alcohol in Rats Fed a Choline Deficient Diet (Takeuchi, Takada, Ebata, Sawae and Okumura, 1968) and *Factors Contributing to Hepatic Cell Necrosis* (Takeuchi, Takada, Kanayama, Ohara and Okumura, 1969).

Since we have already reviewed our studies on the problem elsewhere (Takeuchi and Takada, 1972), we may discuss them very briefly here.

Because alcoholic hepatitis is usually encountered in human chronic alcoholics with pre-existing hepatic damage (such as fatty liver), who have consumed excessive amounts of alcohol recently (Beckett, Livingstone and Hill, 1961), an attempt was made to explore the acute effect of a large dose of alcohol on the fatty livers of rats fed a choline-deficient diet.

One group of female Wistar rats (130-150g, initial body weight) was maintained on a choline-deficient diet as described by György and Goldblatt (1949) (choline-deficient group), for 5 weeks; another group (normal) of similar weight, was kept on a commercial diet for more than 2 weeks. One subgroup of choline-deficient rats and another of normal rats were given a single intoxicating dose of alcohol by gastric intubation (0.75g per 100g of body weight) after fasting for 12h. A second subgroup of choline-deficient rats and one of normal rats, in the same fasting state, were given calorically equivalent amounts of glucose in place of alcohol.

In normal rats, the values of the serum glutamic-oxalacetic transaminase (SGOT), glutamic-pyruvic transaminase (SGPT), and lactic dehydrogenase (SLDH), obtained at various intervals after the administration of alcohol or glucose, showed no significant differences from the values before alcohol or glucose administration. Histologically, no specific alteration of hepatocytes was found in either group, except for the deposition of small fat droplets in the alcohol-treated group.

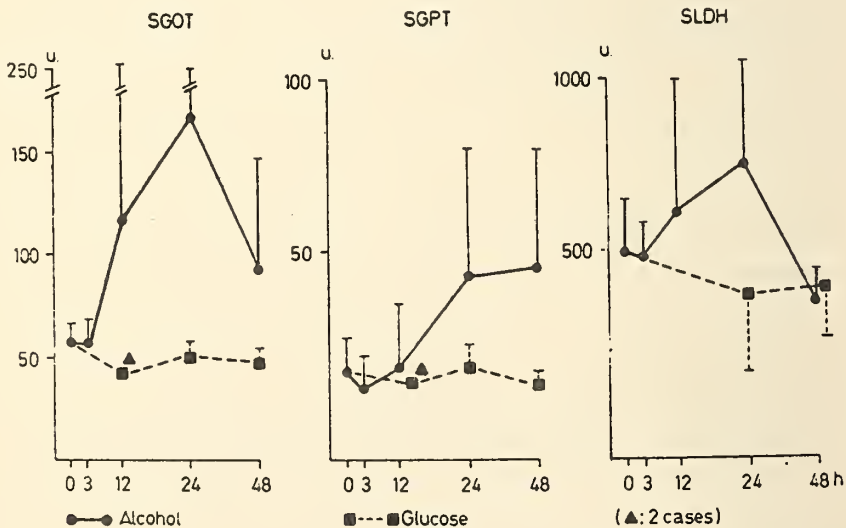


Figure 7. Changes in serum enzymatic activities of choline-deficient rats, following a single dose of alcohol or glucose. SGOT: serum glutamic-oxalacetic transaminase; SGPT: serum glutamic-pyruvic transaminase; SLDH: serum lactic dehydrogenase. Each point at the various intervals represents the mean of 5 to 7 rats, and vertical lines indicate standard deviation of the mean.

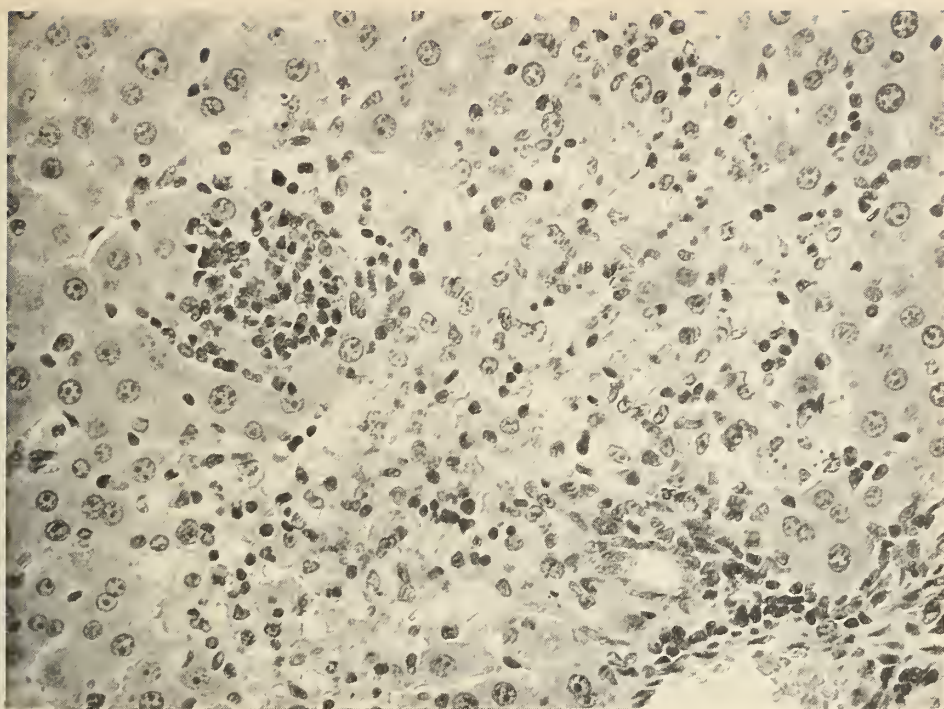


Figure 8. *Photomicrograph of liver of a choline-deficient rat, 24h after a single dose of alcohol. Scattered areas of hepatic cell necrosis are seen. Hematoxylin and eosin $\times 100$.*

In the choline-deficient rats, over half of the animals showed a significant increase of SGOT activities at 24h after alcohol administration. The mean serum enzyme activities at various intervals after alcohol administration are shown in Figure 7. Although the mean activities for SGPT and SLDH were also increased at the same time intervals as for SGOT, the increases were not significant. The three enzyme activities of glucose-treated choline-deficient rats showed no significant changes during the experiment. In 2 out of 10 rats in which SGOT was elevated after alcohol administration, scattered areas of hepatic cell necrosis, associated with leukocytic infiltration, were observed histologically (Fig. 8). In the choline-deficient glucose-treated group, hepatic cell necrosis was not found.

Comment. — These results indicate that even a large amount of alcohol causes neither an elevation of SGOT nor hepatic cell necrosis in normal liver cells. When some pathologic change such as fatty liver is already present, alcohol can produce elevation of SGOT and hepatic cell necrosis. This acute effect of alcohol on the damaged liver of rats may represent a direct hepatotoxic action of alcohol, and is also compatible with the fact that alcoholic hepatitis is usually superimposed upon the already injured liver. Our experiment was the first which produced hepatic cell necrosis in a damaged liver by a single administration of alcohol in the experimental animal. The inflammatory reaction associated with hepatic cell necrosis is weaker in degree compared with alcoholic hepatitis in humans. However, one of the features of alcoholic hepatitis, *i.e.* hepatic cell necrosis, can now be experimentally reproduced in animals.

Although in our experiment the pathogenesis of the elevations of SGOT and hepatic cell necrosis was not clarified, it was shown that many factors, such as the amount of alcohol ingested, the severity of fatty change or the dietary imbalance appeared to contribute to this injurious action of alcohol (Takeuchi *et al.*, 1969).

Hepatic Changes in Chronic Alcoholic Rats Following Periodic Acute Alcoholic Intoxications; Reproducing Hyaline Bodies in the Animals (Takeuchi, Takada, Kato, Hasumura, Ikegami and Matsuda, 1971).

In this experiment, acute doses of alcohol were repeatedly administered by gastric intubation in rats chronically consuming alcohol in the drinking fluid. This schedule of periodic, acute alcohol intoxication superimposed on chronic alcohol ingestion, resembles the periodic, acute alcohol debauches of chronic alcoholic humans.

Sprague-Dawley male rats, 130 to 160g of initial body weight, were divided into two groups. One group was fed with a commercial diet (commercial diet group), and the other with semisynthetic diets (semisynthetic diet group). The compositions of the diets are shown in Table II. Each group of animals was further divided into four subgroups: double loading, single loading, intermittent and nonalcohol groups. The double loading and single loading groups of rats were given 10% glucose-20% alcohol solution as drinking fluid and, in addition, the former group received a single dose of alcohol (0.6g per 100g of body weight) by gastric intubation in the morning once weekly, and the latter group received caloric equivalents of glucose in the same manner. The intermittent and nonalcohol groups of rats were offered tap water as drinking fluid and, in addition, each group of rats received a single dose of alcohol and glucose respectively, in the same manner as described above.

TABLE II

COMPOSITION OF THE COMMERCIAL AND SEMISYNTHETIC DIETS

Commercial diet		Semisynthetic diet	
Ingredient, g/100 g		Alcohol group	Non-alcohol group
Crude protein	26.5	Casein	15.7
Carbohydrate	45.8	Sucrose	56.4
Fat	6.1	Lard	17.6
Cellulose	4.9	Cellulose	1.5
Vitamine powder	3.2	Vitamin powder	4.0
Salt mixture	6.5	Salt mixture	4.0
Water	7.0	L-cystine	0.5
		Choline chloride	0.3
Total	100.0	100.0	100.0

In the semisynthetic diet groups, the two kinds of diets were designed in such a manner that caloric proportions of protein and fat to the total caloric intake were equal between the groups of double and single loading and the groups of intermittent loading and nonalcohol, and that caloric proportions of protein and fat were respectively lower and higher than the corresponding commercial diet groups.

The distribution and daily intake of total calories in each group are shown in Table III. In the commercial diet group, the caloric proportions of protein in the groups of double and single loading were lower than those of the groups of intermittent loading and nonalcohol. However, the proportion of protein (20%) was still higher than that of the semisynthetic diet group.

TABLE III

AVERAGE DAILY INTAKE OF TOTAL CALORIES AND
CALORIES PROVIDED BY ALCOHOL AND PROTEIN

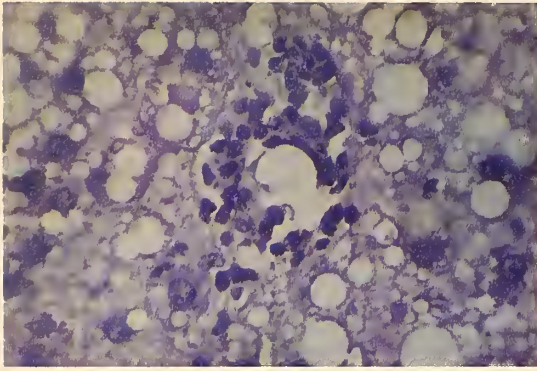
Groups	Number	Total calories	Alcohol	Protein	Fat
Semisynthetic diet					
Double loading	5	65.6 ± 6.3 ^a	22.4 ± 1.6 (34.1%) ^b	9.2 ± 1.2 (14.0%)	23.3 ± 3.0 (35.5%)
Single loading	7	66.7 ± 4.4	23.3 ± 0.9 (34.9%)	9.3 ± 0.8 (13.9%)	23.2 ± 2.2 (34.8%)
Intermittent	5	66.8 ± 2.8		9.4 ± 1.8 (14.1%)	23.6 ± 1.0 (35.3%)
Nonalcohol	4	62.7 ± 3.7		8.8 ± 0.5 (14.0%)	22.1 ± 1.3 (35.2%)
Commercial diet					
Double loading	5	63.8 ± 3.5	21.4 ± 2.2 (33.5%)	13.6 ± 0.5 (21.3%)	5.6 ± 0.7 (8.8%)
Single loading	7	61.6 ± 6.4	21.9 ± 1.7 (35.5%)	13.0 ± 1.4 (21.1%)	5.2 ± 0.7 (8.4%)
Intermittent	4	59.9 ± 4.4		21.4 ± 0.4 (35.7%)	9.1 ± 0.7 (15.2%)
Nonalcohol	5	61.9 ± 5.2		19.8 ± 0.5 (32.0%)	9.4 ± 0.8 (15.2%)

^aValues are expressed as mean ± SD

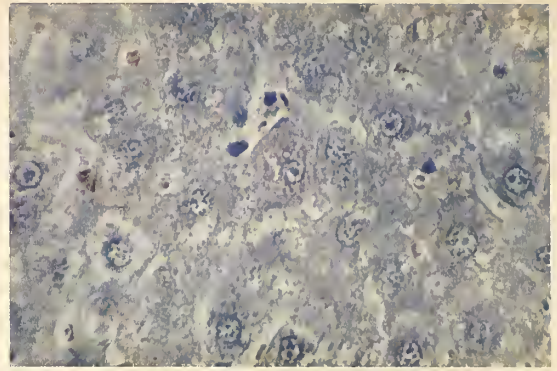
^bNumbers in parentheses refer to caloric proportions of each ingredient.

The degree of fatty liver evaluated by Oil Red O staining sections was more prominent in the groups fed the semisynthetic diets than in those fed the commercial diet. Among the semisynthetic diet groups, the most severe fatty changes were observed in the double loading group, whereas differences in the degree of fatty changes of the liver among the other three groups were not recognizable. In the commercial diet groups the degree of fatty change was prominent in the order of the double loading, single loading, intermittent, and nonalcohol groups.

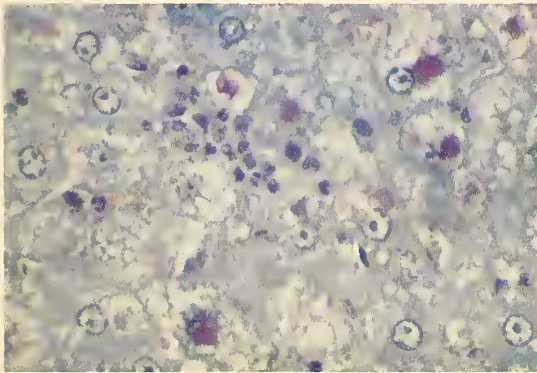
Intracellular hyalin was found abundantly in the semisynthetic diet-double loading group (Table IV). The usual configuration of hyaline bodies was that of rounded paranuclear masses, smaller or equal to the size of nuclei. Some of the hyaline bodies were surrounded by inflammatory cells (Fig. 9a). These hyaline bodies were stained with



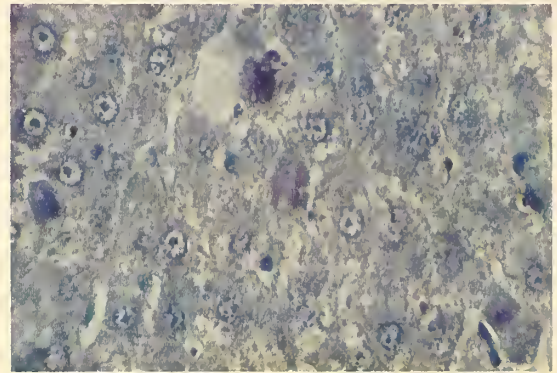
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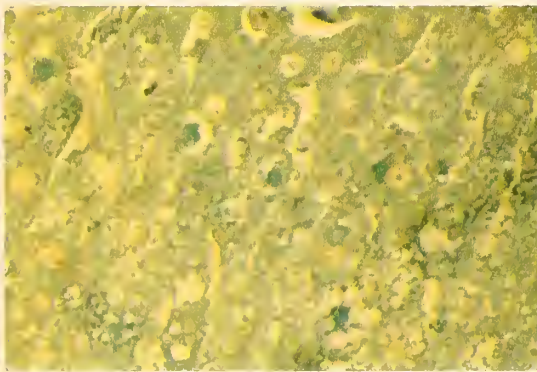
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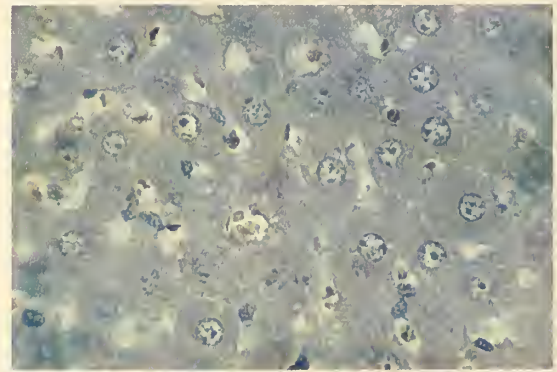
b



e



c



f

Figure 9, a, b, c. Hyaline bodies in hepatocytes of rats from the semisynthetic diet-double loading group. Hyaline bodies in a and b accompanied polymorphonuclear cell infiltration; a Hematoxylin and eosin b Chromotrope aniline blue, c Luxol fast blue. $\times 510$.

d, e, f. Hyaline bodies showing various configurations in hepatocytes of rats from the semisynthetic diet-single loading group e, f, or the commercial diet-double loading group d. Chromotrope aniline blue. $\times 510$.

chromotrope aniline blue, luxol fast blue, and phosphotungstic acid hematoxylin and appeared as large, coarse, aggregated, and compact droplets (Fig. 9, b, c.). In the semi-synthetic diet-single loading group, and the commercial diet-double loading group, intracellular hyalin was observed sporadically. The configuration of hyaline bodies in these groups was fine, granular, discrete, and rodlike or ramified (Fig. 9, d, e, f). No hyaline body was found in the commercial diet-single loading group, in either of the intermittent groups, or the nonalcohol groups.

TABLE IV

INCIDENCE OF HYALINE BODIES

Groups	Number	—	+	++	+++	% Positive
Semisynthetic diet						
Double loading	5	0	1 (1) ^a	2 (1)	2 (1)	100
Single loading	7	4	2	1	0	43
Intermittent	5	5	0	0	0	
Nonalcohol	4	4	0	0	0	
Commercial diet						
Double loading	5	3 (2)	2 (1)	0	0	40
Single loading	7	7	0	0	0	0
Intermittent	4	4	0	0	0	
Nonalcohol	5	5	0	0	0	

^aNumbers in parentheses indicate numbers of dead rats.

Changes other than fatty liver and hyaline bodies were minor even in the double loading groups. Distinct fibrosis or cirrhosis was not found in any rat.

Both of the double loading groups showed steadily higher SGOT activity than the other three corresponding groups, although the differences were not statistically significant, because of the small number of rats in each group.

Comment. — F. B. Mallory spent many years trying to produce changes similar to human Mallory bodies in the experimental animal, without convincing results (Mallory, 1932; Mallory 1960). Hartroft and his group were the first to report the production of Mallory bodies in rats given alcohol chronically (Porta, Hartroft and de la Iglesia, 1965; Gomez-Dumm, Porta, Hartroft and Koch, 1968; Koch, Porta and Hartroft, 1968 and 1969; Porta and Gomez-Dumm, 1968; Porta, Koch and Hartroft, 1969). They also claimed that the variety of tinctorial affinities and histochemical reactions of these hepatocytic deposits was similar to that used in characterization of Mallory's human

alcoholic hyaline bodies, and they did not hesitate in calling the deposits Mallory bodies (Porta *et al.*, 1969). However, Scheig (1970) criticized the published photomicrographs as not resembling Mallory bodies seen in man, although they could be rat Mallory bodies. Iseri and Gottlieb (1971) also claimed that "Mallory bodies" of Hartroft's group did not correspond to the original description of F. B. Mallory by light microscopy.

On light microscopy in our study, hyaline materials, especially of irregular form, closely resemble Mallory bodies in the human. Chey, Kosay, Siplet and Lorber (1971) also report that they produced hyaline bodies, globular or irregular in shape, in dogs administered excessive quantities of alcohol daily for periods ranging from 10 to 18 months. The photomicrographs of their hyaline bodies are similar to ours. There are divergent opinions concerning the ultrastructure of Mallory bodies. One group of investigators believes that the materials are derived from altered mitochondria, (Porta, Bergman and Stein, 1965; Porta *et al.*, 1965; Steiner, Jézéquel, Phillips, Miyai and Arakawa, 1965; Porta and Gomez-Dumm, 1968) and others claim that they originate from focal cytoplasmic degeneration involving both mitochondria as well as lysosomes (Flax and Tisdale, 1964), altered ergastoplasm, (Reppart, Peters, Edmondson and Baker, 1963; Biava, 1964), or fibrillar materials without a limiting membrane (Smuckler, 1968; Iseri and Gottlieb, 1971; Gerber, Orr, Denk, Schaffner and Popper, 1973). Although neither Chey *et al.* (1971) nor we (Takeuchi *et al.*, 1971) investigated the hyaline materials by electron microscopy, it might be said that materials which closely resemble Mallory bodies in humans, at least by light microscopy, can be reproduced in the experimental animals given alcohol.

The pathogenetic role of alcohol in hepatic lesions including Mallory bodies in alcoholic hepatitis is still controversial. Beckett *et al.* (1961) emphasized a direct toxic action of alcohol on the liver. On the other hand, Hartroft and his group claimed that the pathogenesis of hepatic lesions due to chronic alcohol administration in rats was not a direct hepatotoxic effect of alcohol but rather a dietary imbalance induced by alcoholic calories (Gomez-Dumm *et al.*, 1968; Porta and Gomez-Dumm, 1968; Koch *et al.*, 1968 and 1969; Porta *et al.*, 1969). According to these authors, the spontaneous consumption of sweetened alcohol (alcohol plus sucrose) by rats fed solid diets considered normal (for rats not consuming alcohol) creates nutritional imbalances and results in severe liver injury, unless the solid diets are adequately supplemented with lipotropes and vitamins (Koch *et al.*, 1969). In our experiment, hyaline bodies were observed only in double loading and single loading groups of semisynthetic diet group and double loading group of commercial diet group, but not in intermittent and nonalcohol groups of either semisynthetic or commercial diet. These facts indicate that the development of hyaline bodies is not explainable solely by dietary factors, but is related to alcohol administration. The semisynthetic diet group showed more severe fatty liver and more hyaline bodies in the liver than the corresponding commercial diet group, indicating that dietary conditions, such as the low proportion of protein, as well as vitamins and lipotropes (resulting from the dilution by alcohol), and the high proportion of fat, were important in the development of both hyaline bodies and fatty liver.

Green, Mistilis and Schiff (1963) in a clinical study of 50 cases of alcoholic hepatitis, suggested that excessive alcohol consumption and poor nutritional intake might have been etiological factors. Harinasuta, Chomet, Ishak and Zimmerman (1967) claimed that malnutrition, alcohol, and perhaps contaminants, all converge on the hepatocyte to yield the degenerative lesion called Mallory body. Madsen, Bang, Iversen and Jagt (1959) also reported that alcohol had a direct hepatotoxic action in human chronic alcoholics who consumed a poor diet. Although it is now widely accepted from the work of Lieber and

his group (Lieber, Jones and DeCarli, 1965; Lieber and Rubin, 1968; Rubin and Lieber, 1968 a and b) that alcohol, per se, independent of nutritional factors, is a potent hepatotoxic agent, our experimental study supports the opinion that both factors, direct hepatotoxicity of alcohol and nutritional imbalance, might play roles in the pathogenesis of hepatic injury seen in chronic alcoholics.

Which factor, chronic, or periodic acute, intoxication plays a more important role in the pathogenesis of hepatic injury? The effect of repeated acute intoxications of alcohol was evident in the commercial diet groups in which caloric proportions of protein and fat in the total caloric intakes were 21% and 8%, respectively. Jones and Greene (1966) reported that the same levels of protein and fat prevent hepatic changes in rats consuming liquid diets that provide 36% of the calories as alcohol. Our results clearly indicate that repeated debauches of alcohol in addition to chronic consumption of moderate amounts of alcohol might play an important role in the development of severe hepatic lesions. Our experiment also showed that more hyaline bodies, higher SGOT activities, and more severe fatty livers were observed in the double loading groups than in the corresponding single loading groups. As the daily caloric intakes and the caloric proportions of nutrients in the total caloric intakes in the double loading and single loading groups were almost the same, the difference between both groups was attributed to periodic acute alcoholic intoxications.

MECHANISM OF THE DEVELOPMENT OF HEPATIC CELL NECROSIS DUE TO ALCOHOL ADMINISTRATION

We succeeded in producing hepatic cell necrosis by a single dose of alcohol in choline-deficient rats. However, it was very difficult to study the mechanism of hepatic cell necrosis, because the changes in the hepatic cells by choline-deficiency itself were so severe. Recently, during studies on the effect of inducers of microsomal drug metabolizing enzymes upon the alcohol-induced fatty liver (Takada, Matsuda and Ikegami, 1973), we found that some rats pretreated with phenobarbital for 1 week revealed an elevation of serum ornithine carbamyltransferase (OCT) activity and hepatic cell necrosis after a single dose of alcohol. This might be a more simple experimental model for the study on hepatic cell necrosis due to alcohol than the choline-deficient rats. Therefore, we tried to elucidate the mechanism of the development of hepatic cell necrosis due to alcohol, using this experimental model.

Hepatic Changes in the Phenobarbital Pretreated Rats Following Alcohol Administration

Our previous study (Takada *et al.*, 1973) on the effect of phenobarbital on alcohol-induced fatty liver showed that the inhibitory effect of phenobarbital on hepatic triglyceride accumulation after alcohol administration was an apparent one due to dilution by the increased liver masses rather than a true effect. We also found, as mentioned above, an elevation of serum OCT activity and hepatic cell necrosis in a few rats pretreated with phenobarbital for 1 week after the alcohol administration. In this experiment, more long-term treatment with phenobarbital was given to clarify the role of the phenobarbital pretreatment in the development of hepatic cell necrosis due to alcohol administration.

One group of Sprague-Dawley male rats, 150-200g of body weight, was given 0.1% sodium phenobarbital solution as drinking fluid for 5 weeks, and another group of rats received tap water for the same period. Half of each group received a single intoxicating dose of alcohol (0.6g per 100g body weight) and the other half was given an isocaloric amount of glucose by gastric intubation. Changes in serum enzymes and the liver at 16h after the alcohol administration were observed.

Liver weight in the phenobarbital pretreated groups was twice that of the non-pretreated groups. Hepatic triglyceride changes after a single dose of alcohol were the same as those in the previous study pretreated with phenobarbital for 1 week. Protein and cytochrome P-450 contents in the microsomes were significantly higher in the phenobarbital pretreated groups. Cytochrome P-450 tended to be low after alcohol administration in the phenobarbital pretreated groups but the difference between the alcohol and the glucose treated groups was not significant statistically.

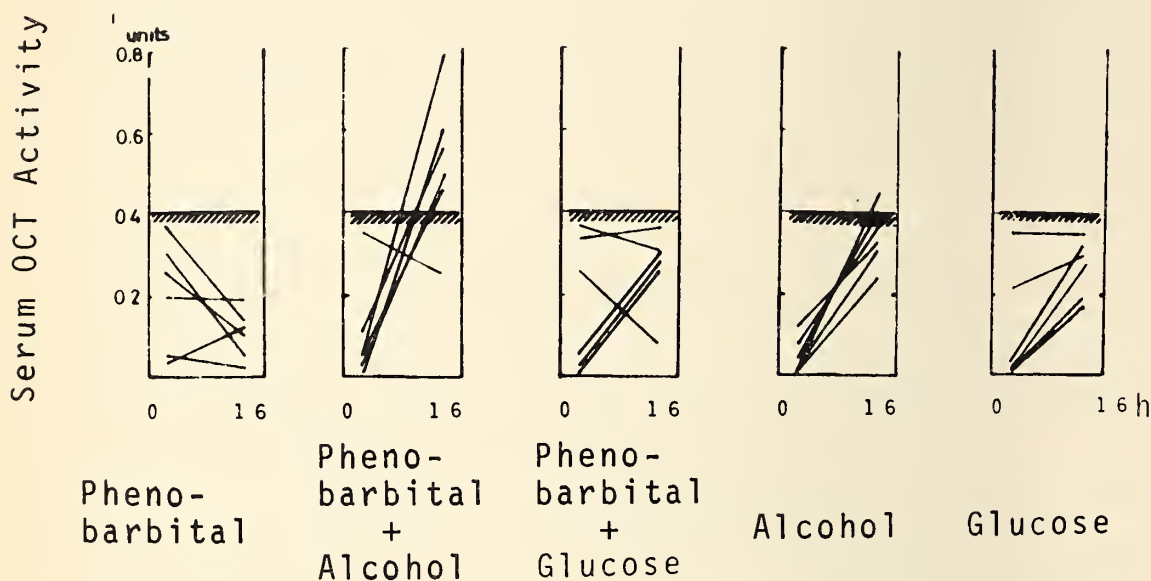


Figure 10. Changes in serum ornithine carbamyl transferase activity at 16h after a single dose of alcohol or glucose in the phenobarbital pretreated or non-pretreated rats.

Significant elevations of serum OCT activity beyond 0.4 units which was the upper limit of normal, were observed in 5 of 6 rats in the phenobarbital pretreated group after the alcohol administration. In the non-pretreated group, serum OCT activity tended to be increased after the alcohol administration in all rats, but an abnormally high value, above 0.4 units, was found in only one rat. Changes in serum OCT activity after the glucose administration were limited to under 0.4 units (Fig. 10).

On light microscopy, enlarged hepatic cells were observed around the hepatic veins in the phenobarbital pretreated groups. Very slight fatty change of the hepatic cells in the central and midzonal areas was found in almost all of the rats, and the degrees of fatty changes were not different among the groups. Small foci of hepatic cell necrosis around the central vein were found in a rat of the phenobarbital pretreated, alcohol administered group (Fig. 11).

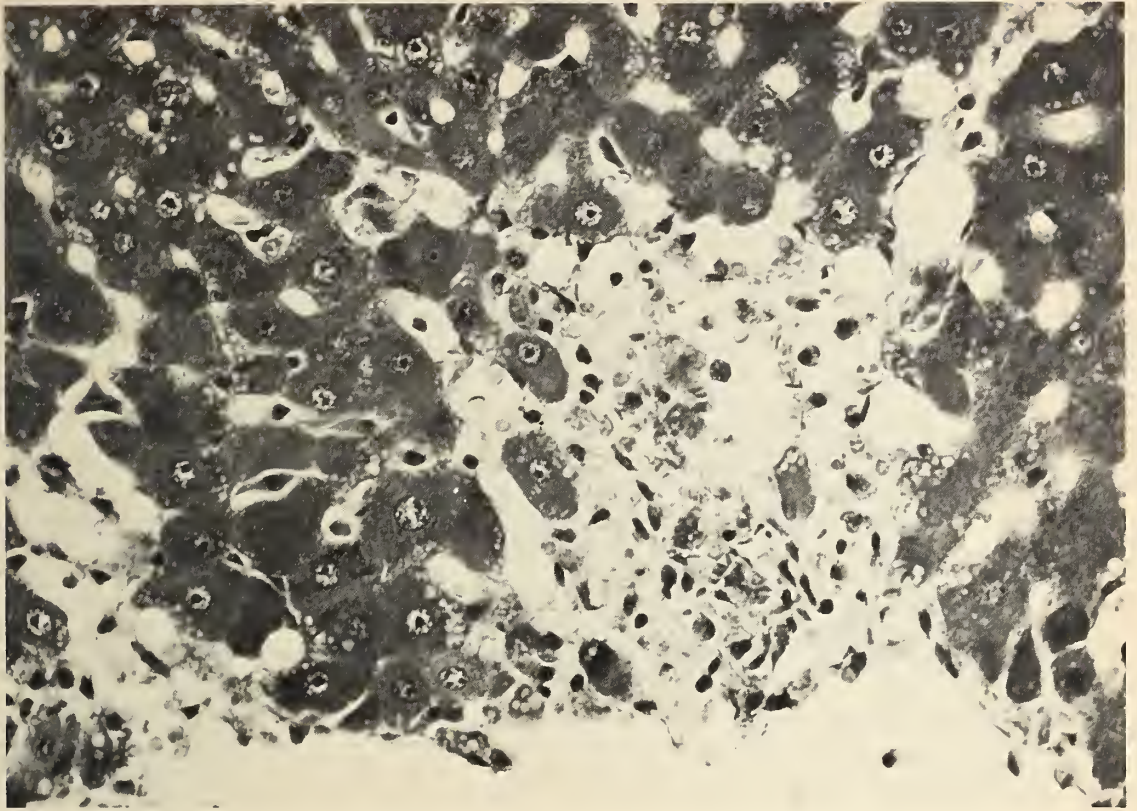


Figure 11. *Photomicrograph of the liver of a phenobarbital pretreated rat after a single dose of alcohol. Focal necrosis of the hepatocytes is seen around the hepatic vein. Hematoxylin and eosin x300.*

On electron microscopy prominent proliferation of smooth endoplasmic reticulum (ER) was found in the phenobarbital pretreated groups. In the non-pretreated group, large, distorted or bizarre mitochondria and fat droplets were observed after the alcohol administration. These changes were less prominent in the phenobarbital pretreated group. The most characteristic alterations in the phenobarbital pretreated group after the alcohol administration were marked distension of the cisternae of the rough ER, detachment of the ribosomes of the dilated rough ER and an increase in number of autophagic vacuoles (Figs. 12 and 13). These changes were observed only in the phenobarbital pretreated, alcohol administered rats.

Comment. — Hepatic cell necrosis was found in one rat only, after alcohol administration, suggesting that serum OCT elevation resulted from more subtle alterations of membrane permeability. The increased number of autophagic vacuoles indicates focal cytoplasmic degeneration. Dilatation of the cisternae of the rough ER and detachment of the ribosomes is one of the characteristic electron microscopic features of acute viral hepatitis (Tanikawa, 1968). These results indicate that, under our experimental condition, alcohol can induce degenerative alterations of the micro-organelles of the hepatic cells which are similar to those of acute hepatitis.

In previous experiments (Takada *et al.*, 1973), only 3 of 9 rats pretreated with phenobarbital for 1 week showed significant elevations of serum OCT activity (a specific index of hepatocellular injury) after a single dose of alcohol. However, a significant

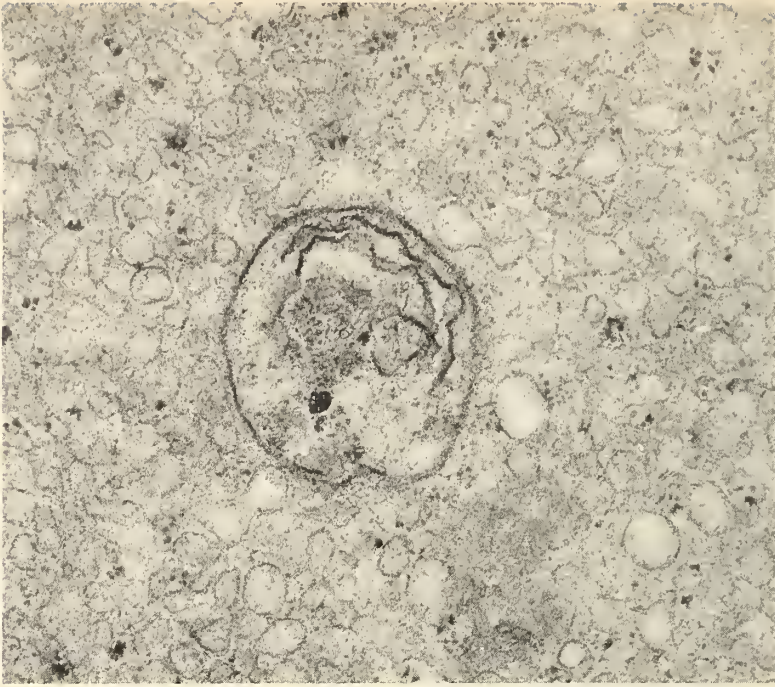


Figure 12. *Electron micrograph of a hepatocyte of a phenobarbital pretreated rat after a single dose of alcohol. Prominent proliferation of smooth ER and autophagic vacuole are seen. $\times 20,000$.*

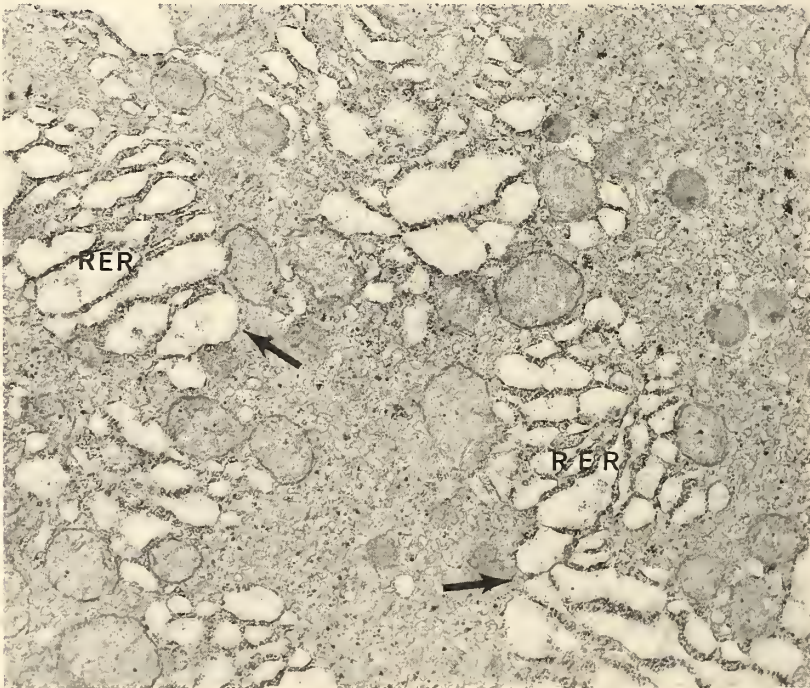


Figure 13. *Electron micrograph of the same rat of Fig. 11. Marked distension of the cisternae of the rough endoplasmic reticulum (RER) and detachment of the ribosomes of the dilated rough ER (\uparrow) are seen. $\times 20,000$.*

elevation of serum OCT activity after the alcohol administration was found in 5 of 6 rats pretreated with phenobarbital in the present experiments. These results suggest that changes in serum OCT activity induced by alcohol become more prominent after long-term pretreatment with phenobarbital in which microsomal proliferation may be more prominent. This indicates that intense proliferation of the microsomes might be one of the conditions of the hepatic cell which make it vulnerable to alcohol toxicity. There are two possible explanations of the vulnerability of the hepatic cells to alcohol. The first is an increased rate of alcohol metabolism in the microsome due to intense induction of drug metabolizing enzymes, and the second, development of the hypertrophic hypoactive endoplasmic reticulum due to the long-term pretreatment of phenobarbital.

Effect of Phenobarbital on Alcohol Metabolism

The previous experiment suggested that an increased rate of alcohol metabolism in the microsome might be a factor in the development of hepatic cell necrosis due to alcohol. However, the results of several studies (Fisher, 1962; Khanna and Kalant, 1970; Koff, Carter, Lui and Isselbacher, 1970; Mezey, 1971; Lieber and DeCarli, 1972) on the interaction of alcohol and barbiturate metabolism are controversial. In this experiment, blood alcohol and phenobarbital clearances were studied in rats treated with phenobarbital to clarify the relationship of alcohol and barbiturate metabolism, especially the relationship of enzyme induction in the microsome by phenobarbital and the metabolism of alcohol in the microsome.

One group of rats was given 0.1% of sodium phenobarbital as drinking fluid for 1 week (phenobarbital-no interval: PB-O group), one group of rats received tap water for 2 days following pretreatment with phenobarbital for 1 week (phenobarbital: PB group) and the other group of rats was given tap water for 9 days (control group); blood phenobarbital level before the alcohol administration was $9.10 \pm 2.20 \mu\text{g/ml}$ in the phenobarbital-no interval (PB-O) group, but was trace in the phenobarbital (PB) group. A series of experiments was performed to observe, firstly the effect of blood barbiturate level on alcohol metabolism. Blood alcohol clearance was compared between two control groups of rats administered alcohol (0.2g per 100g of body weight) intraperitoneally with or without simultaneous administration of pentobarbital (4mg per 100g of body weight). Alcohol was administered, in the same manner, to rats of PB-O and PB groups and blood alcohol clearance was also compared between the two groups. Secondly, the effect of alcohol on pentobarbital metabolism was noted. Pentobarbital (4mg per 100g of body weight) was given to each control group of rats with or without alcohol administration (0.2g per 100g of body weight) intraperitoneally, and blood pentobarbital clearances were compared in both groups (rats of control group administered alcohol and pentobarbital simultaneously in the experiments 1 and 2 are the same animals). Thirdly, the effect was observed of a single dose of pyrazole (30 mg per 100g of body weight), which is a potent inhibitor of alcohol dehydrogenase in the cytosole. This was given to each 5 rats of the phenobarbital and control groups by gastric intubation 3 hours before the alcohol administration to evaluate the function of microsomal ethanol oxidizing system (MEOS).

In the first experiment (Fig. 14), the blood half-life of alcohol was significantly shorter in the control group administered pentobarbital simultaneously than that of the control-alcohol alone group; on the other hand, there was no significant difference in the half-life of alcohol between PB-O and PB groups. In the second experiment (Fig. 15),

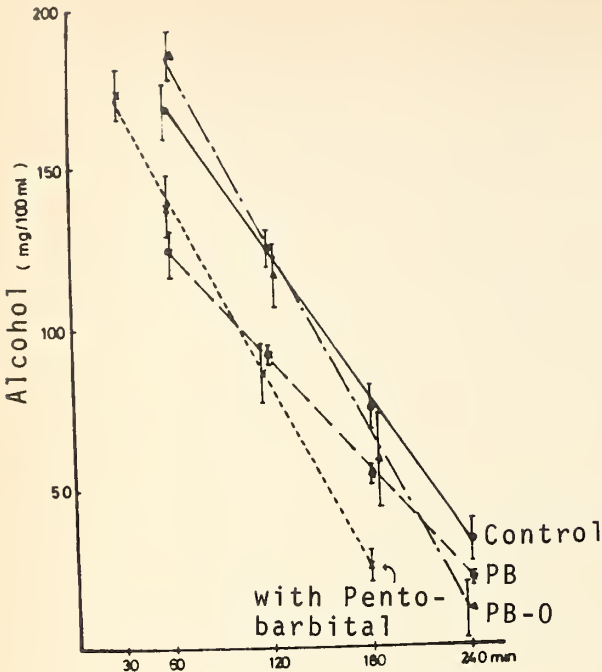


Figure 14. Blood alcohol levels after a single injection of alcohol intraperitoneally in the rats pretreated with or without phenobarbital for 1 week. Control: no pretreatment with phenobarbital, with pentobarbital: simultaneous administration with pentobarbital in the control group. PB-O: phenobarbital for 1 week, no interval, PB: phenobarbital for 1 week, 48 hours interval. In this and following figures, vertical lines indicate standard error of the mean.

Figure 15. Blood pentobarbital clearance after a single injection of pentobarbital with or without alcohol administration.

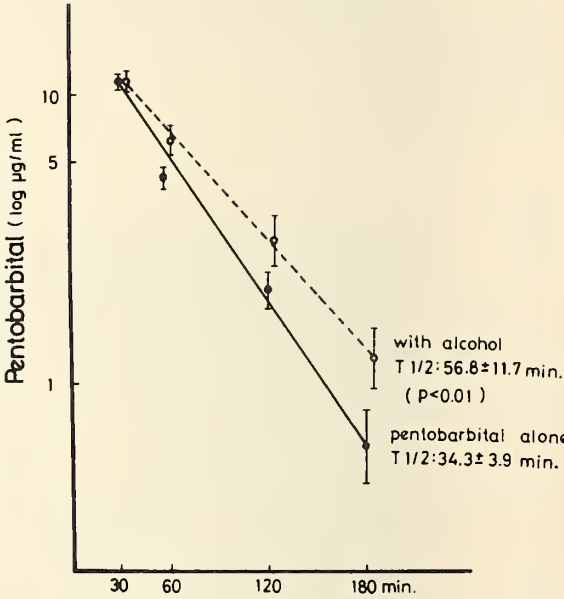
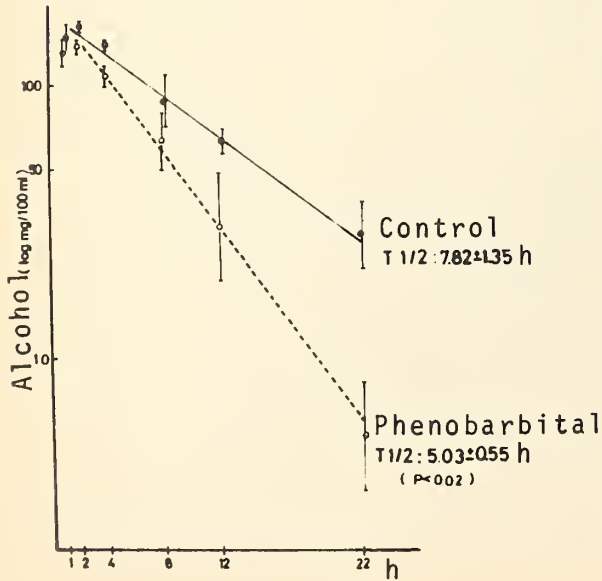


Figure 16. Blood alcohol clearance after a single dose of alcohol in the rats of the control and phenobarbital groups pretreated with pyrazole.

blood pentobarbital clearance was significantly prolonged by the simultaneous administration of alcohol compared with single administration of pentobarbital. In the third experiment, the half-life of alcohol in the phenobarbital group was significantly shorter than that of the control group after the pyrazole treatment (Fig. 16).

Liver weight, microsomal protein and cytochrome P-450 contents were significantly increased in the phenobarbital treated group compared with the control group. MEOS activity expressed per mg of protein was not different between the two groups. However, total MEOS activity was significantly increased in the phenobarbital group (Table V).

TABLE V

CYTOCHROME P-450 CONTENT AND MEOS ACTIVITY IN THE RATS TREATED WITH PHENOBARBITAL FOR 1 WEEK. (MEAN \pm S.D.)

Group		Phenobarbital	Control
No. of rats		5	5
Liver weight	g/100g of body weight	4.74 ± 0.15 ($p < 0.001$)	3.08 ± 0.21
Microsomal protein	mg/g liver	13.94 ± 0.49 ($p < 0.001$)	10.26 ± 1.12
Cytochrome P-450	m μ mole/mg protein	4.96 ± 0.94 ($p < 0.01$)	1.52 ± 0.64
	m μ mole/g liver	69.3 ± 14.5 ($p < 0.001$)	14.6 ± 4.9
MEOS	μ g/min/mg protein	2.47 ± 0.21	2.42 ± 0.54
	μ g/min/g liver	34.4 ± 4.0 ($p < 0.01$)	24.4 ± 4.0
	μ g/min/100g of body weight	162.9 ± 12.4 ($p < 0.001$)	75.0 ± 12.3

$p < 0.01$ and $p < 0.001$: compared to respective controls

Comment. — In the present experiments, the disappearance rate of pentobarbital from the blood was significantly decreased by the simultaneous administration of alcohol. This is in agreement with previous reports (Rubin, Gang, Misra and Lieber, 1970; Lieber and DeCarli, 1972) and suggests substrate interaction of alcohol and barbiturate with the microsomal drug metabolizing enzyme system. On the other hand, disappearance rate of alcohol from the blood was accelerated by the simultaneous administration of pentobarbital and was not influenced by the pretreatment with phenobarbital for 1 week. Thus, the effect of chronic or acute administration of barbiturate on the metabolism of alcohol by induction of drug metabolizing enzymes or interaction between the two substrates in the hepatic microsome was not detected by an ordinary clearance test, because approximately only a quarter of alcohol may be metabolized in the microsome.

However, the half-life of alcohol after the pretreatment with pyrazole, which is a potent inhibitor of alcohol dehydrogenase but not of MEOS, was significantly shortened in the phenobarbital group. This suggests that the metabolism of alcohol through the MEOS may be accelerated in the phenobarbital group. MEOS activity in the microsome preparation was not significantly increased by treatment with phenobarbital for 1 week when it was expressed per mg of protein. However, the activity was significantly increased when it was expressed per total liver, because of a significant increase in microsomal protein. The same results were also reported by Mezey (1971) and Lieber and De Carli (1972). This means that an increase in MEOS activity following the barbiturate treatment may not be detected by an *in vitro* study. Total hepatic activity of MEOS may be reflected only by *in vivo* study such as the blood alcohol clearance test after the pyrazole treatment.

The entity of MEOS or the role of MEOS *in vivo* is still controversial (Khanna, Kalant and Lin, 1970; Lieber, Rubin and DeCarli, 1970; Carter and Isselbacher, 1971). Apart from the character of MEOS, our results suggest that microsomes play some role in alcohol metabolism *in vivo*, and alcohol metabolism in the microsome is accelerated by the pretreatment with phenobarbital. Since acetaldehyde dehydrogenase is not localized in the microsome, an increase in alcohol metabolism in the microsome would result in accumulation of acetaldehyde in microsomes. Although acetaldehyde concentration in the microsome has not yet been estimated, the enhancement of alcohol metabolism in the microsome might make the hepatic cell vulnerable to alcohol, because of the intense toxicity of acetaldehyde.

Microsomal Changes After Long-Term Treatment of Phenobarbital

In the previous experiment, it was suggested that the intense proliferation of microsomes, especially the development of a hypertrophic hypoactive endoplasmic reticulum, might be a factor in the development of hepatic cell necrosis due to alcohol. Although it has been well known that phenobarbital is an intense inducer of the drug metabolizing enzymes, the effect of long-term treatment of phenobarbital on hepatic microsomes is not known. In this experiment, changes in the hepatic microsome following long-term treatment with phenobarbital were studied to explore the possibility of the development of hypertrophic hypoactive endoplasmic reticulum.

One group of rats was given 0.1% sodium phenobarbital as drinking water, and another group of rats received tap water as control. Each group of rats was sacrificed at intervals of 3 days and 1, 3, 5, and 10 weeks and the serial changes of hepatic microsome were observed. Some rats of each group were given a single dose of alcohol (0.6g per 100g of body weight) or an isocaloric amount of glucose at the 5th week, and changes in the microsome were studied.

Liver weights and microsomal protein of the liver were significantly higher in the phenobarbital treated group than the control group (Fig. 17). Cytochrome P-450, aniline hydroxylase, and hexobarbital hydroxylase activity in the microsome were also increased in the phenobarbital treated group when expressed per gram of the liver (Fig. 18). When expressed as mg of microsomal protein, cytochrome P-450 and b-5 content showed persistently high values, while drug metabolizing enzymes showed peak values at the third day or the first week and then gradually decreased through the experimental period. Microsomal phospholipid content and MEOS activity did not show significant changes, while microsomal RNA content and glucose-6-phosphatase activity showed somewhat lower values (Fig. 19 and 20). These results indicate that composition of the hepatic

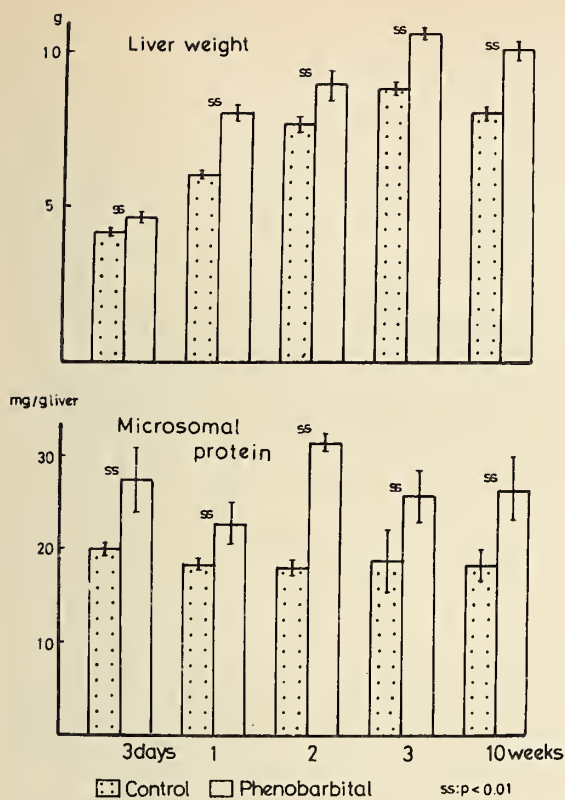
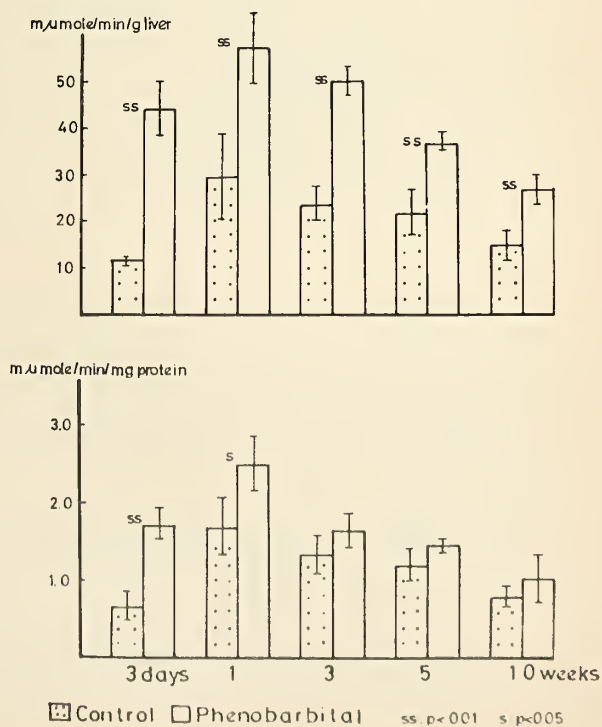


Figure 17. Liver weight and microsomal protein in the phenobarbital treated and control groups.

Figure 18. Hexobarbital hydroxylase activity in the microsome in the phenobarbital treated and control groups.



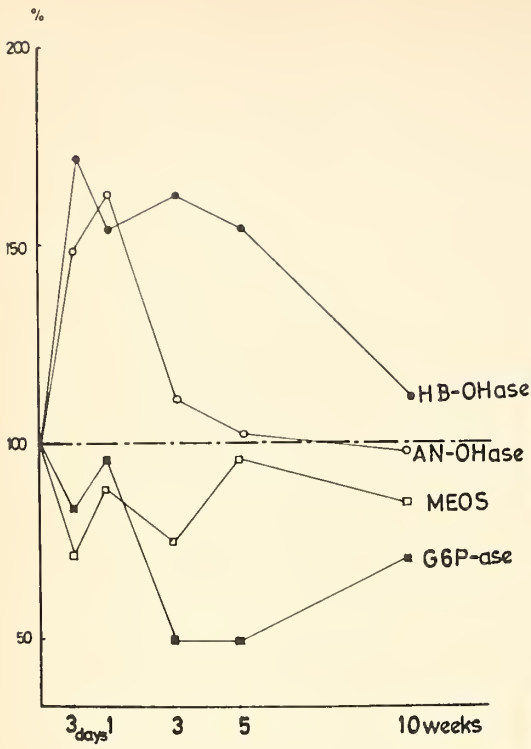


Figure 19. Percent changes from the control values in activities (expressed per mg of microsomal protein) of hexobarbital hydroxylase, aniline hydroxylase, glucose-6-phosphatase and ethanol-oxidizing system in the microsome after phenobarbital administration. HB-OHase: hexobarbital hydroxylase, AN-OHase: aniline hydroxylase, MEOS: microsomal ethanol-oxidizing system, G6P-ase: glucose-6-phosphatase.

microsome changed greatly after long-term treatment with phenobarbital. Although drug metabolizing enzymes were increased by the phenobarbital treatment when expressed per gram of the liver, the activity per mg of microsomal protein gradually decreased with the course of phenobarbital treatment, suggesting the development of hypertrophic hypoactive endoplasmic reticulum.

After a single dose of alcohol, hepatic OCT activity was decreased only in the phenobarbital treated group. This result corresponded with an elevation of serum OCT activity. Glucose-6-phosphatase activity tended to be increased in the non-treated group and to be decreased in the phenobarbital group after a single dose of alcohol; the difference of the activity between both groups was statistically significant, although difference from the corresponding group administered isocaloric amount of glucose was insignificant statistically (Fig. 21).

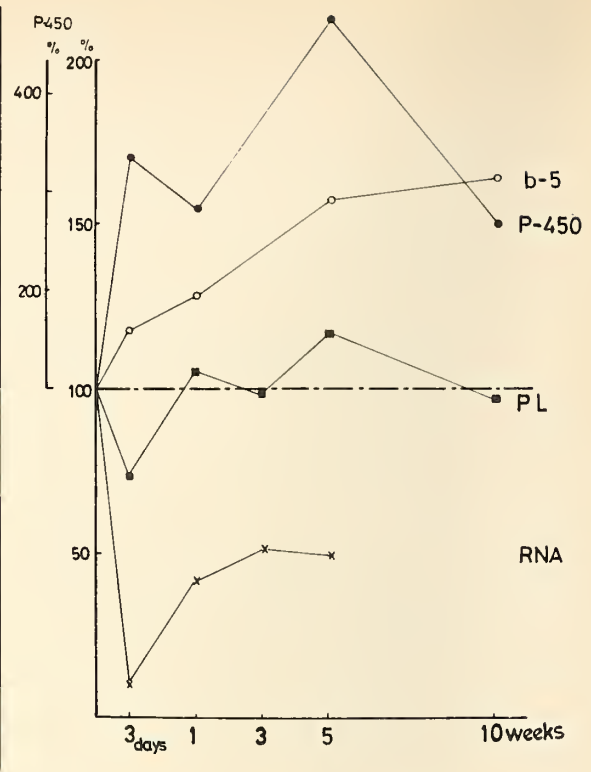


Figure 20. Percent changes from the control values in the content (expressed per mg of microsomal protein) of phospholipids, cytochrome P-450 and b-5, and ribonucleic acid in the microsome after phenobarbital administration. P-450 and b-5: Cytochrome P-450 and b-5, RNA: ribonucleic acid, PL: phospholipids.

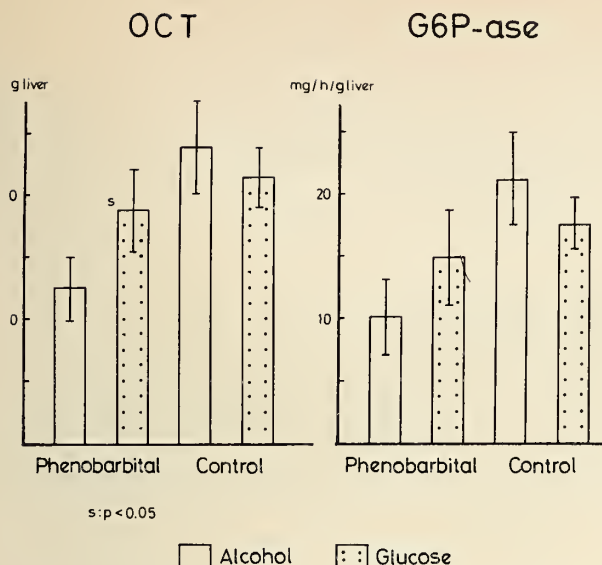


Figure 21. Changes of ornithine carbamyl transferase and glucose-6-phosphatase activities in the liver after a single dose of alcohol or isocaloric amount of glucose at the 5th week. Phenobarbital: phenobarbital treated group, Control: phenobarbital non-treated group.

Comment. — Many drugs induce different microsomal changes according to their hepatotoxicity. Feuer, Cooper, de la Iglesia and Lumb (1972) reported that hepatotoxic agents such as carbon tetrachloride decrease drug metabolizing enzyme activity as well as microsomal RNA content and glucose-6-phosphatase activity. Non-hepatotoxic agents such as phenobarbital increase only drug metabolizing enzymes. Methyl cholanthrene or ethionine, which are weak hepatotoxic agents, increase drug metabolizing enzymes and decrease RNA content and glucose-6-phosphatase activity. Changes in microsome after alcohol administration in the phenobarbital treated group in the present experiment were similar to those produced by ethionine as reported by Feuer *et al.* (1972). These results also offer biochemical evidence for hepatotoxicity of alcohol.

Hutterer, Schaffner, Klion and Popper (1968) reported that long-term treatment with dieldrin resulted in a hypertrophic hypoactive endoplasmic reticulum which is a transitional form from adaptation to injury. The same pattern was observed following long-term treatment with phenobarbital. These results suggest that intense or abnormal proliferation of the microsome might be one of the conditions which make the hepatic cells vulnerable to alcohol toxicity.

SUMMARY

Epidemiological investigation of alcoholic cirrhosis in Japan reveals that alcoholic cirrhosis has been rapidly increasing. This increase parallels increases in the annual intake of alcohol and dietary fat, suggesting the pathogenetic role of both factors in the development of alcoholic cirrhosis.

A single dose of a large amount of alcohol produces elevation of SGOT activity and hepatic cell necrosis in choline-deficient rats but not in normal rats, suggesting that alcohol can induce hepatic cell necrosis when other pathologic changes are already present. Hyaline bodies could be also produced in rats by alcohol administration. The production of hyaline bodies and other changes were all greater in the rats consuming alcohol chronically and acutely (double loading group) than in the rats consuming it only chronically.

(single loading group). Dietary factors played some role in the development of liver injury in association with direct hepatotoxic effect of alcohol.

Hepatic cell necrosis was observed in the phenobarbital treated rats after a single dose of alcohol. There are two possibilities to explain this: (1) enhancement of alcohol metabolism in the microsome and (2) the development of hypertrophic hypoactive endoplasmic reticulum.

ACKNOWLEDGEMENTS

Some Figures and Tables reported in this chapter were taken from Takeuchi *et al.* (1968) and Takeuchi *et al.* (1971). We are indebted to the editors of Laboratory Investigation and the American Journal of Clinical Nutrition for permission to reproduce these data.

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Effect of Alcohols on Various Forms of Chemically Induced Liver Injury

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HISTORICAL

It is well known that individuals exposed to both ethanol and carbon tetrachloride (CCl_4) are more susceptible to the organ-damaging properties of CCl_4 (Von Oettingen, 1964). The potentiating effects of ethanol on the hepatotoxic and nephrotoxic responses to several halogenated hydrocarbons has been shown in experimental animals (Rosenthal, 1930; Stewart, Torkelson, Hake and Erley, 1960; Klaassen and Plaa, 1966, 1967; Kutob and Plaa, 1972).

Since the early investigations consisted of the simultaneous administration of both agents, the explanation proposed was that ethanol facilitates the absorption of the hydrocarbon (Rosenthal, 1930; Stewart *et al.*, 1960), thereby increasing the amount of hydrocarbon being delivered to the tissues. However, individuals who have ingested ethanol several hours before their exposure to the hydrocarbons are also more susceptible (Guild, Young and Merrill, 1958). Kutob and Plaa (1962) showed that in mice, ethanol pretreatment 15 or 24 hours before chloroform exposure resulted in enhanced liver injury, yet the blood had been cleared of ethanol by 9 hours. Therefore, it is clear that the enhanced response occurs not only if ethanol is present, but even when it is no longer present. Although the mechanism involved is unknown, the potentiation is seen to be maximal when the alcohol pretreatment occurs 16 to 18 hours prior to solvent exposure (Traiger and Plaa, 1971).

Several other aliphatic alcohols have been shown to exert a potentiating effect on the hepatotoxicity of CCl_4 (Cornish and Adeliuin, 1967). Using serum glutamic-oxalacetic transaminase as an index of hepatotoxicity, these investigators demonstrated that a number of alcohols are even more effective potentiators than ethanol. Isopropanol

was remarkably effective. Table I summarizes data which show the potentiating abilities of various aliphatic alcohols in mice. The isopropanol effect is the most striking.

The work to be described in this paper deals primarily with the potentiation observed with isopropanol. In addition to studies carried out with CCl_4 , other hepatotoxic agents were employed to test the specificity of the isopropanol interaction.

TABLE I
POTENTIATING EFFECT OF ALCOHOLS ON CCl_4 TOXICITY IN MICE

Treatment	Dose (ml/kg)	CCl_4	SGPT Units
Methanol	10	No	45
		Yes	296*
Ethanol	7	No	52
		Yes	84*
n-Propyl	1.75	No	60
		Yes	63
Isopropanol	2.5	No	58
		Yes	2147*
n-Butyl	3	No	43
		Yes	50
4-Methyl-2-pentanol	1.4	No	55
		Yes	79

The alcohol was given p.o. 20 hours before the CCl_4 (0.0075 ml/kg, i.p.). Liver function was assessed 24 hours after CCl_4 . Data obtained from Traiger and Plaa, 1971. (n=6).

*Significantly different from group given alcohol alone, $p < 0.05$.

METHODS

Male Sprague-Dawley rats weighing 150-280 grams were used throughout. The animals were maintained on an *ad libitum* diet of commercial chow and water. Various halogenated hydrocarbons were employed: CCl_4 , chloroform, 1, 1, 2-trichloroethane, trichloroethylene and 1, 1, 1-trichloroethane. These substances were administered by the intraperitoneal route. In other experiments, beryllium sulfate was given intravenously, galactosamine was given intraperitoneally, allyl alcohol was given orally, and alpha-naphthylisothiocyanate (ANIT) was given orally.

Hepatotoxicity was assessed by elevation in serum glutamic-pyruvic transaminase (SGPT) activity (Reitman and Frankel, 1957), increased hepatic triglyceride concentrations (Butler, Maling, Horning and Brodie, 1961), decreased hepatic glucose-6-phosphatase activity (Traiger and Plaa, 1971), elevation in serum bilirubin concentrations (Indacochea-Redmond and Plaa, 1971), or increased isocitric dehydrogenase (ICD) activity (Witschi and Aldridge, 1967). With the halogenated hydrocarbons, allyl alcohol, galactosamine and ANIT parameters were measured 24 hours after administration of the test substance. With beryllium, varying time periods were assessed.

Comparison of Ethanol and Isopropanol

The effect of ethanol or isopropanol pretreatment on CCl_4 hepatotoxicity is illustrated in Table II. It is evident that the two alcohols themselves had no significant effect on SGPT activity, triglyceride accumulation or glucose-6-phosphatase activity. However, when pretreatment with either of these alcohols occurred 18 hours before the administration of the challenge dose of CCl_4 (0.1 ml/kg) the response to the CCl_4 was greatly increased; this was evident using all three parameters of hepatotoxicity. The challenge dose of CCl_4 merely caused a slight hepatotoxic response when it was given alone. Furthermore, it should be noted that increasing the challenge dose of CCl_4 10-fold (1.0 ml/kg) resulted in a response which was less than that produced with isopropanol plus CCl_4 (0.1 ml/kg). Ethanol plus CCl_4 resulted in a response which is about equal to the response exerted by the 10-fold dose of CCl_4 given alone.

TABLE II

EFFECT OF ALCOHOL PRETREATMENT ON CCl_4 -INDUCED HEPATOTOXICITY IN RATS

Treatment	SGPT (units/ml)	Triglycerides (mg/g liver)	Glucose-6-phosphatase activity (mg/Pi/g liver/20 min)
Ethanol, 5.0 ml/kg	50	8	6.7
Isopropanol, 2.5 ml/kg	50	6	6.2
CCl_4 , 0.1 ml/kg	100	9	6.0
CCl_4 , 1.0 ml/kg	500	17	3.8
Isopropanol + CCl_4 0.1 ml/kg	2250*	22*	2.8*
Ethanol + CCl_4 , 0.1 ml/kg	500*	13*	4.8*

The alcohol was given p.o. 18 hours before the i.p. CCl_4 . Liver function was assessed 24 hours after CCl_4 . Data obtained from Traiger and Plaa, 1971. (n=10).

*Significantly different from group given alcohol alone, $p < 0.05$.

Studies were conducted to see if the pretreatment time interval (the time between the alcohol administration and the CCl_4 administration) affected the interaction. These data are summarized in Table III. With ethanol, potentiation of the SGPT response occurred if the CCl_4 was given 18 hours or less after the alcohol. However, with isopropanol, potentiation still occurred if 24 hours intervened between the two treatments. Measuring triglyceride accumulation or depressed glucose-6-phosphatase activity, enhanced responses occurred with both alcohols when the pretreatment time interval was 24 hours. With both alcohols, maximal potentiation occurred when the CCl_4 was given 18 hours after the alcohol.

Isopropanol produced a more marked potentiation of CCl_4 than did ethanol, and yielded quantitatively greater responses in all parameters measured. On a molar basis, the

TABLE III

EFFECT OF THE PRETREATMENT INTERVAL ON CCl₄-INDUCED HEPATOTOXICITY IN RATS

Pretreatment Interval (hours)	Potentiated Hepatotoxic Response					
	SGPT (units/ml)		Triglycerides (mg/g liver)		Glucose-6-Phosphatase (mgPi/g liver/20 min)	
	Isopropanol	Ethanol	Isopropanol	Ethanol	Isopropanol	Ethanol
3	550*	200*	14*	13*	4.1*	5.0*
8	1150*	475*	17*	15*	3.2*	4.1*
18	2250*	500*	22*	13*	2.8*	4.7*
24	2300*	75	21*	11*	4.0*	5.0*
48	75	—	14*	—	6.7	6.2*

The alcohol was given p.o. at varying time intervals before the CCl₄ (0.1 ml/kg, i.p.). Liver function was assessed 24 hours after CCl₄. Data obtained from Traiger and Plaa, 1971. (n=10).
*Significantly different from group given alcohol alone, p < 0.05.

dose of ethanol employed (5.0 ml/kg) is equal to 0.09 mol/kg, whereas that of isopropanol (2.5 ml/kg) is equal to 0.03 mol/kg. This makes the increased response with isopropanol even more striking. When the dose of ethanol was reduced to 2.5 ml/kg, the potentiation of CCl₄ was no longer evident. However, with isopropanol, it was possible to lower the dose to 0.41 ml/kg and still elicit the enhancement of the CCl₄ response (Plaa and Traiger, 1973).

The maximal responses obtained with CCl₄ and isopropanol cannot be explained as mere additive effects since the values obtained for the combination were always greater than the sum of the responses from either agent alone.

Parent Alcohol versus Metabolic Product

Because of the marked quantitative differences between ethanol and isopropanol pretreatment on the subsequent response to the challenge dose of CCl₄, experiments were carried out to determine whether it were the unmetabolized alcohol which was responsible for these responses or whether it were the metabolic degradation product. Pyrazole (150 mg/kg, i.p.) was utilized to block the oxidation of the two alcohols; this inhibitor of alcohol dehydrogenase was administered 15 minutes before pretreatment with the alcohol. Pyrazole markedly inhibited the elimination of ethanol. Normally, 8 hours after ethanol, the blood ethanol concentration was less than 25 mg/100 ml. After pyrazole, the concentrations at 8, 18 and 24 hours were 225, 100 and 50 mg/100 ml, respectively. The data in Table IV show that pyrazole administration resulted in a further potentiation of the CCl₄ response. Using all three parameters of hepatotoxicity, it was possible to show that those animals pretreated with pyrazole showed a much more enhanced response to the CCl₄ challenge than did those animals treated only with ethanol. SGPT activities after ethanol plus CCl₄ were 14 per cent of that found for pyrazole plus ethanol plus

CCl₄. From a quantitative standpoint, pyrazole plus ethanol plus CCl₄ yielded a potentiated response which was similar to that seen after isopropanol plus CCl₄. Therefore, with ethanol it appears that it is the unmetabolized alcohol which is responsible for the potentiated response to CCl₄.

TABLE IV

EFFECT OF PYRAZOLE ON ALCOHOL-POTENTIATED RESPONSES TO CCl₄ IN RATS

Treatment	SGPT (units/ml)	Triglycerides (mg/g liver)	Glucose-6-phosphatase activity (mgPi/g liver/20 min)
Pyrazole	90	6	5.4
Pyrazole + CCl ₄	137	8	4.4*
Pyrazole + Ethanol	85	8.5*	5.6
Ethanol + CCl ₄	430*	16.8*	4.9*
Pyrazole + Ethanol + CCl ₄	3263†	20*	2.8†
Pyrazole + Isopropanol	77	8.8	6.3
Isopropanol + CCl ₄	2604*	23.6*	2.9*
Pyrazole + Isopropanol + CCl ₄	1118†	9.5†	3.4*

The pyrazole (150 mg/kg, i.p.) was given 15 min. before the alcohol. The alcohol was given 18 hours before the CCl₄ (0.1 ml/kg, ip). Liver function was assessed 24 hours after CCl₄. Data obtained from Traiger and Plaa, 1972. (n=10).

*Significantly different from controls, $p < 0.05$.

†Significantly different from alcohol plus CCl₄, $p < 0.05$.

With isopropanol, the response was quite different. Pyrazole reduced the potentiating effects of isopropanol, using all three parameters of response. This unexpected observation indicated that isopropanol was metabolized to another substance which was capable of potentiating CCl₄ hepatotoxicity. It was possible to demonstrate (Table V) that normally isopropanol is metabolized to acetone and that pyrazole inhibits the formation of acetone. Therefore, the pyrazole studies suggested that the acetone formed from isopropanol may be contributing to the enhanced CCl₄-induced hepatotoxicity observed after treatment with isopropanol.

In order to demonstrate that acetone itself could produce enhanced responses to CCl₄, animals were pretreated with this substance (1 ml/kg, p.o.) and subsequently challenged with CCl₄. It was demonstrated (Table VI) that acetone itself could enhance the hepatotoxic response to CCl₄ using SGPT activity and glucose-6-phosphatase activity as parameters of hepatotoxicity. In the same study, acetaldehyde was also tested although there was no evidence that this metabolic degradation product of ethanol was involved in the potentiation by ethanol. The data in the Table show that acetaldehyde had no enhancing effect on CCl₄ hepatotoxicity.

TABLE V

EFFECT OF PYRAZOLE ON THE ELIMINATION OF ISOPROPANOL AND FORMATION OF ACETONE IN BLOOD AFTER ADMINISTRATION OF ISOPROPANOL TO RATS

Time (hours)	Control		After Pyrazole	
	Isopropanol (mg/100 ml)	Acetone (mg/100 ml)	Isopropanol (mg/100 ml)	Acetone (mg/100 ml)
0.75	85	8	—	—
1	75	21	—	—
3	67	83	87	23*
10	29	92	45*	46*
18	4	73	50*	54*
40	—	—	3	15

Pyrazole (150 mg/kg, i.p.) was given 15 min. before the isopropanol (2.5 ml/kg, p.o.). Isopropanol and acetone in blood were determined by gas chromatography (Traiger and Plaa, 1972). Data obtained from Plaa and Traiger, 1973. (n=3).

*Significantly different from control, $p < 0.05$.

The question then arose as to which component acetone or unchanged isopropanol was exerting the major effect in the potentiation of CCl_4 . From the data in Table V, it is seen that pyrazole increased the concentration of isopropanol 11-fold at 18 hours, while reducing acetone concentrations by 27 per cent; yet a diminished potentiation to isopropanol was observed (Table IV). These results tend to suggest an important role for acetone.

TABLE VI

EFFECT OF ACETONE AND ACETALDEHYDE ON CCl_4 -INDUCED HEPATOTOXICITY IN RATS

Treatment	SGPT (units/ml)	Glucose-6-phosphatase (mgPi/g liver/20 min)
Acetone	53	6.3
CCl_4	82*	5.2*
Acetone + CCl_4	810†	4.2*
Acetaldehyde	49	7.0
CCl_4	63	5.1
Acetaldehyde + CCl_4	63	5.4

Acetone or acetaldehyde (1.0 ml/kg, p.o.) was given 18 hours before the CCl_4 , (0.1 ml/kg, p.o.). Liver function was assessed 24 hours after CCl_4 . Data obtained from Traiger and Plaa, 1972. (n=10).

*Significantly different from control, $p < 0.05$.

†Significantly different from group given CCl_4 alone, $p < 0.05$.

An experiment was performed in which isopropanol metabolism to acetone was retarded by giving pyrazole. The animals were then treated with both isopropanol and acetone before being challenged with CCl_4 . The results are summarized in Table VII. Pyrazole treatment in animals receiving only isopropanol resulted in a diminution of the enhanced response to CCl_4 , as seen before (Table IV). If the pyrazole-treated rats received both isopropanol and acetone simultaneously, a greater enhancement of the CCl_4 response was observed. From Table V, it is evident that peak acetone concentrations in the blood occur around 10 hours after the administration of isopropanol. If the acetone treatment was given 10 hours after isopropanol and pyrazole, the potentiation was even more striking; the CCl_4 response in this group of rats was indistinguishable from that observed in rats treated only with isopropanol and receiving no pyrazole. It should be noted that pyrazole does not affect acetone elimination *in vivo* (Traiger and Plaa, 1972). These experiments support the hypothesis that newly formed acetone plays a major role in the isopropanol- CCl_4 interaction.

TABLE VII

EFFECT OF ACETONE ON ISOPROPANOL POTENTIATION OF CCl_4
HEPATOTOXICITY IN RATS

Treatment and Times			SGPT (units/ml)
4 PM	2 AM	10 AM	
Isopropanol	—	CCl_4	3584
Pyrazole + Isopropanol	—	CCl_4	644*
Pyrazole + Isopropanol + Acetone	—	CCl_4	1656*
Pyrazole + Isopropanol	Acetone	CCl_4	3166

Pyrazole (150 mg/kg, i.p.) was given 15 min. before isopropanol (2.5 ml/kg, p.o.). Acetone dose was 1.0 ml/kg, p.o. Liver function was assessed 24 hours after CCl_4 . Data obtained from Traiger and Plaa, 1972. (n=5).

*Significantly different from isopropanol plus CCl_4 .

Another approach was utilized to assess the relative importance of acetone and isopropanol. From elimination studies carried out in normal rats, it was calculated that about 85 per cent of the isopropanol administered was converted to acetone. Two dose-response studies were performed using isopropanol or acetone treatment alone, 18 hours before challenging the animals with CCl_4 . The data in Table VIII show that the potentiating properties of both agents were about equal when the substances were administered in comparable doses. The dose-response curves derived from these data were virtually superimposable. These experiments also support the hypothesis that the metabolite acetone plays a major role in the isopropanol- CCl_4 interaction.

This series of experiments indicates that with ethanol the observed potentiation can be attributed to the unaltered ethanol. This is consistent with the observed prolongation of ethanol elimination and the enhancement of ethanol-induced potentiation of CCl_4 by pyrazole. The inability of acetaldehyde to elicit a potentiated response would also ascribe a major role to unaltered ethanol.

TABLE VIII

EFFECT OF VARYING DOSES OF ACETONE OR ISOPROPANOL
ON CCl₄ HEPATOTOXICITY IN RATS

Isopropanol		Acetone	
Dose (ml/kg)	SGPT (units/ml)	Dose (ml/kg)	SGPT (units/ml)
0.41	800	0.35	525
1.18	900	1.00	1400
2.94	2000	2.50	1500
4.70	2400	4.00	2500

The isopropanol or acetone was given p.o. 18 hours before CCl₄ (0.1 ml/kg, i.p.). Liver function was assessed 24 hours after CCl₄. Data obtained from Plaa and Traiger, 1973.

With isopropanol, the data indicate that the potentiation cannot be attributed to the alcohol alone. Pyrazole, which prolongs isopropanol elimination from the blood, reduces the potentiating capacity of isopropanol. Acetone, the metabolic product of isopropanol, is also capable of augmenting the CCl₄ response. The addition of acetone to the combination of isopropanol plus pyrazole, at a time coincident with depression of peak acetone concentrations by pyrazole, overcomes the inhibitory action of pyrazole. Therefore, acetone production is involved in the isopropanol-CCl₄ interaction. The marked differences in potentiating capacity exhibited between ethanol and isopropanol can most likely be due to the oxidation of ethanol to a metabolite which does not augment CCl₄ hepatotoxicity, whereas the oxidation of isopropanol results in the production of a metabolite which can affect CCl₄ hepatotoxicity.

TABLE IX

EFFECT OF PYRAZOLE ON THE INTERACTION OF 1-BUTANOL
AND CCl₄ IN RATS

Treatment	SGPT (units/ml)
Control	57
1-Butanol + CCl ₄	81*
Pyrazole + 1-butanol + CCl ₄	1688†

Pyrazole (150 mg/kg, i.p.) was given 15 min. before 1-butanol (2.0 ml/kg, p.o.). The 1-butanol was given 18 hours before CCl₄ (0.1 ml/kg, i.p.). Liver function was assessed 24 hours after CCl₄. Data obtained from Traiger and Plaa, 1972.

*Significantly different from control, $p < 0.05$.

† Significantly different from 1-butanol plus CCl₄, $p < 0.05$.

Ethanol is not the only aliphatic alcohol which seems to potentiate CCl_4 by way of the unchanged alcohol. 1-Butanol did not enhance CCl_4 hepatotoxicity when given 20 hours before CCl_4 in mice (Table I). The same absence of effect occurred in rats (Table IX). However, if rats were pretreated with pyrazole before being subjected to 1-butanol, a marked potentiation of CCl_4 was observed.

Role of Hepatic Microsomal Enzymes

There is a considerable body of evidence which indicates that CCl_4 is activated before it exerts its acute hepatotoxic response (Slater, 1966; Recknagel and Ghoshal, 1966; Reynolds and Yee, 1967, Gordis, 1969). Since it appears that the activation of CCl_4 occurs in the endoplasmic reticulum, several groups have studied the effects of enzyme inducers on CCl_4 toxicity. Garner and McLean (1969) showed that phenobarbital resulted in enhanced metabolism of CCl_4 to CO_2 and that the hepatotoxic response to CCl_4 was greatly enhanced. Stenger, Miller and Williamson (1970) and Stenger and Johnson (1971) showed that the enhanced toxicity to CCl_4 exerted by phenobarbital paralleled the increase in hepatic drug metabolizing enzymes. Phenobarbital pretreatment resulted in increased lipid peroxidation after CCl_4 (Rao, Glende and Recknagel, 1970). Thus, it appears that microsomal enzyme inducers can potentiate CCl_4 toxicity. The microsomal enzymes might also be involved in the potentiation observed after alcohol treatment.

The catalase inhibitor, aminotriazole, markedly depresses the activities of the drug metabolizing enzymes and cytochrome P-450 content in liver microsomes; this substance inhibits the induction of microsomal oxidases and increases in cytochrome P-450 content produced by phenobarbital (Kato, 1967; Baron and Tephly, 1969). Therefore, a series of experiments was performed to see if this agent could alter the isopropanol-acetone- CCl_4 interaction.

TABLE X

EFFECT OF AMINOTRIAZOLE ON THE POTENTIATION OF CCl_4 BY ISOPROPANOL IN RATS

Treatment	SGPT (units/ml)	Triglycerides (mg/g liver)
Control	71	10.3
Aminotriazole	65	9.0
Aminotriazole + CCl_4	84	13.1*
Isopropanol + CCl_4	3598*	19.7
Aminotriazole + Isopropanol + CCl_4	1202†	13.1*

Aminotriazole (1.0 g/kg, i.p.) was given 15 min. before isopropanol (2.5 ml/kg, p.o.). CCl_4 (0.1 ml/kg, i.p.) was given 18 hours later. Liver function was assessed 24 hours after CCl_4 . Data obtained from Traiger and Plaa, 1973. (n=5).

*Significantly different from control, $p < 0.05$.

†Significantly different from isopropanol plus CCl_4 , $p < 0.05$.

Aminotriazole (1.0 g/kg, i.p.) given 15 minutes before isopropanol or acetone exerted no effect on the disappearance of isopropanol and acetone or on the formation of acetone from isopropanol (Traiger and Plaa, 1973). However, pretreatment with aminotriazole did significantly reduce the potentiation of CCl_4 seen after giving isopropanol (Table X) or acetone (Table XI).

TABLE XI

EFFECT OF AMINOTRIAZOLE ON THE POTENTIATION OF CCl_4 BY ACETONE
IN RATS

Treatment	SGPT (units/ml)	Triglycerides (mg/g liver)
Control	54	8.8
CCl_4	52	8.9
Acetone + CCl_4	1410*	21.1*
Aminotriazole + Acetone + CCl_4	334†	10.6†

Aminotriazole (1.0 g/kg, i.p.) was given 15 min. before acetone (1.0 ml/kg, p.o.). CCl_4 (0.1 ml/kg, i.p.) was given 18 hours later. Liver function was assessed 24 hours after CCl_4 . Data obtained from Traiger and Plaa, 1973. (n=6).

*Significantly different from control, $p < 0.05$.

†Significantly different from acetone plus CCl_4 , $p < 0.05$.

Isopropanol treatment has been shown to cause an increase in hepatic aniline hydroxylase activity when this enzyme was assayed *in vitro* in microsomes derived from livers of pretreated rats (Table XII). Isopropanol causes proliferation of the smooth endoplasmic reticulum of the hepatocyte; this effect occurs sometime between 18 and 42

TABLE XII

EFFECT OF ISOPROPANOL ON HEPATIC ANILINE HYDROXYLASE ACTIVITY IN RATS

Treatment	Aniline Hydroxylase (μmol p-aminophenol/g liver/hr)
Control	0.78
Isopropanol	1.28*
Aminotriazole	0.45*
Aminotriazole + Isopropanol	0.76

Aminotriazole (1.0 g/kg, i.p.) was given 15 min. before isopropanol (2.5 ml/kg, p.o.). Enzyme activity was measured 17 hours after isopropanol. Data obtained from Traiger and Plaa, 1973. (n=3).

*Significantly different from control, $p < 0.05$.

hours (Côté, Traiger and Plaa, 1974). The increased aniline hydroxylase activity is consistent with this change in ultrastructure. Aminotriazole treatment *in vivo* inhibits the isopropanol-induced enhancement of aniline hydroxylase (Table XII).

Preliminary results suggest that a circadian rhythm exists in the potentiation exhibited by ethanol and isopropanol (Plaa and Traiger, 1973). Maximum potentiation caused by ethanol seems to occur if the alcohol is present in high concentrations during the naturally occurring period of maximum microsomal activity described by Radzialowski and Bousquet (1967). However, the data available to date are not yet conclusive and more experiments are needed in this area.

In their entirety, the available evidence suggests strongly that the microsomal enzymes are somehow involved in the alcohol-CCl₄ interaction. Isopropanol can enhance aniline hydroxylase activity (Table XII). Recently, acetone treatment *in vivo* has been found to enhance the microsomal N-demethylation of dimethylnitrosamine (Sipes, Stripp, Krishna, Maling and Gillette, 1973). On the other hand, enhanced microsomal activity does not seem to be the entire explanation. Pretreatment with isopropanol converts the response to the challenging dose of CCl₄ (0.1 ml) to one which is greater than that obtained with a 10-fold increase in dose of CCl₄ (1.0 ml) given alone (Table II). This suggests that there must be another explanation for this marked response to the challenge dose of CCl₄. It is feasible that isopropanol could very well stimulate drug metabolizing enzymes, but could also act on the endoplasmic reticulum in such a way that the attack of CCl₄ (or. CCl₃) on this organelle is facilitated.

Interaction with Other Halogenated Hydrocarbons

The question arises as to whether the potentiating effect of isopropanol is specific to CCl₄ or can occur with other halogenated hydrocarbons. A study very similar to those which have been described in the rat was carried out in mice (male, Swiss Webster, 25-35

TABLE XIII

EFFECT OF ISOPROPANOL ON OTHER HALOGENATED HYDROCARBONS IN MICE

Treatment	Dose (ml/kg)	Isopropanol Pretreatment	SGPT (units/ml)
Chloroform	0.50	No	86
	0.50	Yes	3582*
Trichloroethylene	1.5	No	29
	1.5	Yes	245*
1,1,2-Trichloroethane	0.15	No	117
	0.15	Yes	197*
1,1,1-Trichloroethane	2.5	No	82
	2.5	Yes	98

The isopropanol (2.5 ml/kg, p.o.) was given 18 hours before the halogenated hydrocarbon. Liver function was assessed 24 hours after the hydrocarbon was given. Data obtained from Traiger and Plaa, 1974. (n=5-9).

*Significantly different from group receiving no isopropanol, $p < 0.05$.

grams). The mice were given isopropanol 18 hours before an intraperitoneal dose of chloroform, trichloroethylene, 1, 1, 2-trichloroethane or 1, 1, 1-trichloroethane. In these experiments, only SGPT was used as a measure of hepatic function. The data are summarized in Tables XIII and XIV. It can be seen that isopropanol was capable of potentiating the hepatotoxic response to chloroform and also that of trichloroethylene. However, the pretreatment had very little effect on the other two halogenated hydrocarbons.

TABLE XIV

EFFECT OF ISOPROPANOL ON CHLOROFORM-INDUCED HEPATOTOXICITY IN MICE

Dose (ml/kg)	Isopropanol Treatment	SGPT (units/ml)
0	No	22
	Yes	21
0.05	No	32
	Yes	83*
0.10	No	51
	Yes	252*
0.20	No	60
	Yes	626*
0.50	No	86*
	Yes	3532*

Isopropanol (2.5 ml/kg, p.o.) was given 18 hours before i.p. chloroform. Liver function was assessed 24 hours after chloroform. Data obtained from Traiger and Plaa, 1974. (n=5-10).
*Significantly different from group receiving no isopropanol, $p < 0.05$.

These data indicate that the isopropanol potentiating effect occurs only with those halogenated hydrocarbons which already have a fairly high hepatotoxic potential. The most marked potentiation was seen with chloroform, an agent which is capable of producing moderate to severe liver injury at low doses. Isopropanol only enhanced slightly the effects of 1, 2, 2-trichloroethane and had no effect on 1, 1, 1-trichloroethane. While isopropanol pretreatment caused an increase in the response to trichloroethylene, it was necessary to employ a dose of trichloroethylene in the lethal range to elicit the response.

Acetone pretreatment also resulted in enhanced responses similar to those observed with isopropanol. These data are summarized in Table XV. They indicate that halogenated hydrocarbons other than CCl_4 can be affected by isopropanol and acetone. However, the degree of potentiation observed seems to be related to the hepatotoxic properties of the halogenated hydrocarbon involved. Prior treatment with isopropanol or acetone does not convert a weak hepatotoxic agent (like 1, 1, 1-trichloroethane) into a potent one.

Isopropanol on Other Hepatotoxic Substances

It was of interest to determine whether only the halogenated hydrocarbons could be potentiated by isopropanol. Different types of hepatotoxic agents were utilized. Beryl-

lium sulphate produces midzonal necrosis; allyl alcohol produces periportal necrosis and galactosamine produced focal necrosis. The effect of isopropanol on these agents was assessed in rats.

Serum ICD activity was used for assessing beryllium hepatotoxicity. In one experiment, varying doses of beryllium were administered intravenously and the animals were sacrificed 4 hours later. These data are shown in Figure 1. It is evident that a dose-response relationship could be demonstrated for elevation in ICD following beryllium administration. When the rats were pretreated with isopropanol 18 hours before beryllium ($39 \mu\text{mol/kg}$), there was no potentiation of the beryllium response. A second experiment was carried out in which the rats were sacrificed 24 hours after the administration of beryllium. From Figure 2 it is again evident that a dose-response relationship existed between the elevation in serum ICD and the dose of beryllium. However, in this situation also, pretreatment with isopropanol 18 hours before beryllium did not result in enhanced hepatotoxicity, using two different doses of beryllium (7 and $17 \mu\text{mol/kg}$).

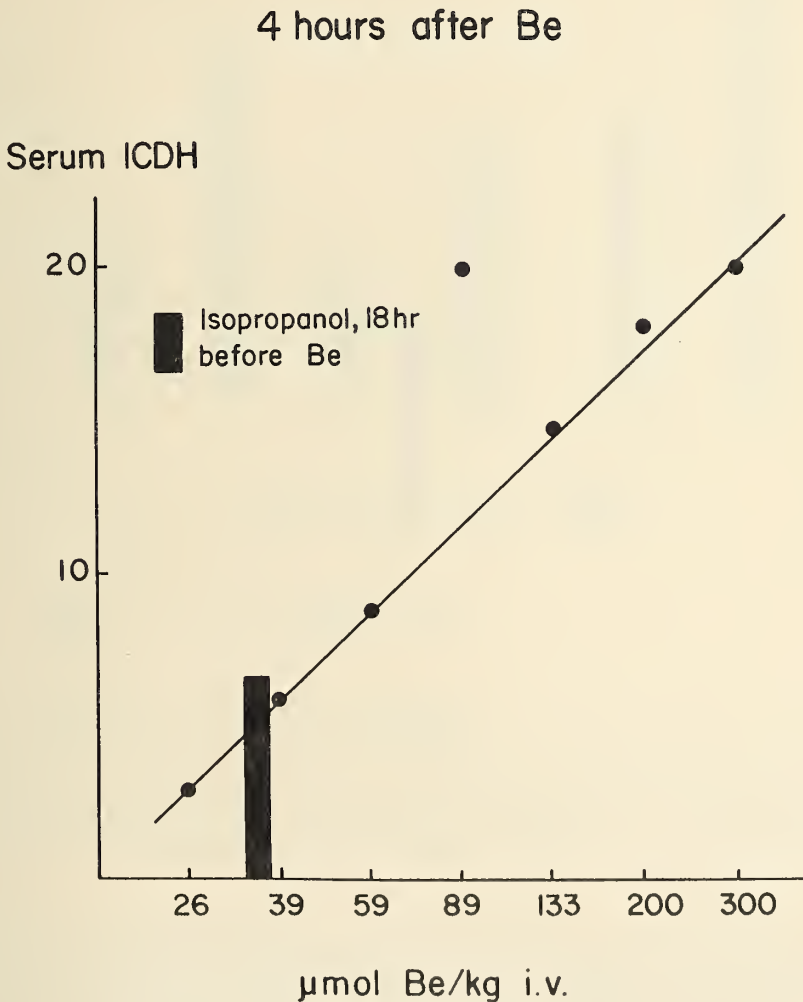


Figure 1. Effect of isopropanol on beryllium-induced increases in serum ICD activity 4 hours after the administration of beryllium. The closed circles denote the responses measured when beryllium was given alone. The bar denotes the response measured when isopropanol (2.5 ml/kg , *p.o.*) was given 18 hours before beryllium. ($n = 6-8$).

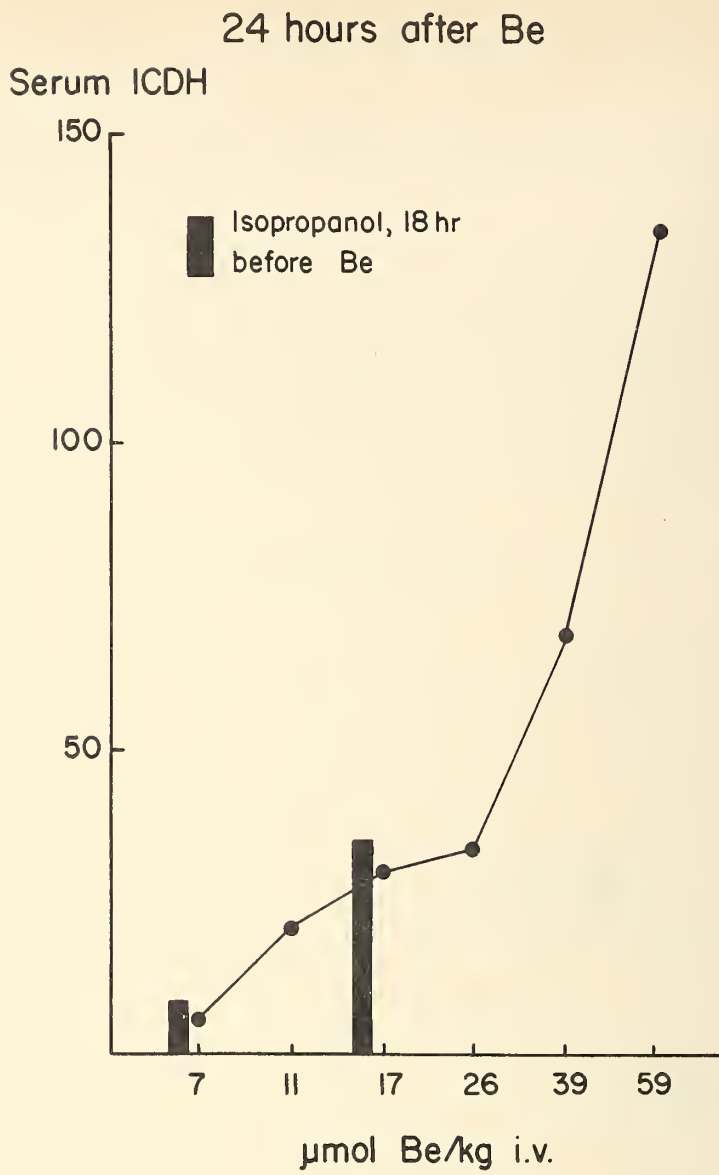


Figure 2. *Effect of isopropanol on beryllium-induced increases in serum ICD activity 24 hours after the administration of beryllium. The closed circles denote the responses measured when beryllium was given alone. The bars denote the responses measured when isopropanol (2.5 ml/kg, p.o.) was given 18 hours before beryllium. (n = 6-8).*

Similar experiments were carried out with allyl alcohol. Serum ICD activity was used to assess hepatic function. Allyl alcohol was given orally and hepatic function was tested 24 hours later. Using six different doses of allyl alcohol, it was possible to show that a reasonably good dose-response relationship occurred for elevation in ICD activity. When isopropanol (2.5 ml/kg p.o.) was given 18 hours before allyl alcohol, no potentiation was observed with the three different doses (10, 20 and 40 mg/kg) of allyl alcohol employed (Figure 3).

With galactosamine, SGPT activity was used to assess hepatic function 24 hours later. Isopropanol (2.5 ml/kg, p.o.) pretreatment 18 hours before galactosamine (200 and 400 mg/kg, i.p.) failed to potentiate the galactosamine response (Table XVI). In fact, the SGPT values for the combination isopropanol-galactosamine (400 mg/kg) were apparently lower than those obtained with galactosamine (400 mg/kg) alone.

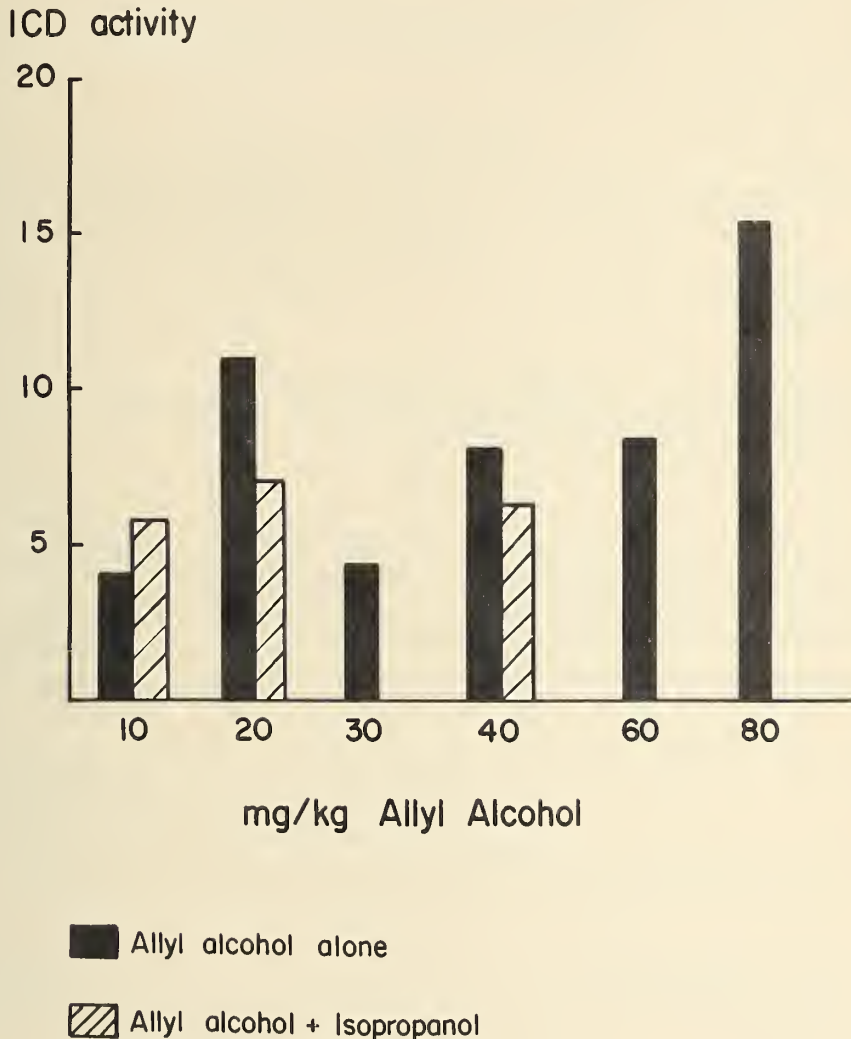


Figure 3. Effect of isopropanol on allyl alcohol-induced increases in serum ICD activity 24 hours after the administration of allyl alcohol.

It is seen that isopropanol was not capable of potentiating the hepatotoxic responses to these three different necrogenic agents. If the isopropanol effect on CCl_4 injury merely reflected a nonspecific increase in hepatocyte sensitivity to any necrogenic agent, a potentiation with beryllium, allyl alcohol or galactosamine would have been expected. Since this did not occur, it seems evident that the isopropanol effect is relatively specific. Furthermore, if isopropanol treatment merely results in the enhanced uptake of chemicals by the hepatocyte, one could also expect that beryllium, galactosamine or allyl

TABLE XV

EFFECT OF ACETONE ON OTHER HALOGENATED HYDROCARBONS IN MICE

Treatment	Dose (ml/kg)	Acetone Treatment	SGPT (units/ml)
Chloroform	0.10	No	42
		Yes	422*
Trichloroethylene	1.5	No	28
		Yes	47
1,1,2-Trichloroethane	0.10	No	26
		Yes	80*
1,1,1-Trichloroethane	2.5	No	130
		Yes	157*

The acetone (1.0 ml/kg, p.o.) was given 18 hours before the halogenated hydrocarbon. Liver function was assessed 24 hours after the hydrocarbon was given. Data obtained from Traiger and Plaa, 1974. (n=4-6).

*Significantly different from group given no acetone, $p < 0.05$.

alcohol would be potentiated. This was not observed; therefore a general effect of isopropanol on tissue uptake of chemicals does not seem likely.

In terms of relative specificity, only those agents which act predominantly on centrilobular cells were the ones found to be potentiated by isopropanol. This is in keeping with the hypothesis that somehow the microsomal activity of the endoplasmic reticulum

TABLE XVI

ISOPROPANOL PRETREATMENT ON GALACTOSAMINE HCl-INDUCED
CHANGES IN SGPT ACTIVITY IN RATS

Treatment	SGPT Activity (units/ml)
Control	51
Isopropanol	53
Galactosamine HCl, 200 mg/kg	103*
Isopropanol + Galactosamine HCl, 200 mg/kg	85*
Galactosamine HCl, 400 mg/kg	219*
Isopropanol + Galactosamine HCl, 400 mg/kg	78*

Isopropanol (2.5 ml/kg, p.o.) was given 18 hours before galactosamine (200 or 400 mg/kg, i.p.). Liver function was assessed 24 hours after galactosamine. (n=6-8).

*Significantly different from control, $p < 0.05$.

is involved. It has been reported that centrilobular cells are relatively rich in some microsomal enzymes and that periportal cells are relatively poor (Wattenberg and Leong, 1962; Koudstall and Hardouk, 1969). Also, while it is possible to potentiate CCl_4 (acting on centrilobular cells) with phenobarbital (Garner and McLean, 1969), allyl alcohol (acting on periportal cells) hepatotoxicity is not potentiated by phenobarbital (Reid, Cho, Krishna and Brodie, 1970), although both hepatotoxic agents are thought to act through a biotransformation product. Perhaps the link between these differences is the relative mixed function oxidase activity of the various hepatic cells.

TABLE XVII

EFFECT OF ISOPROPANOL PRETREATMENT ON ANIT-INDUCED HYPERBILIRUBINEMIA
IN RATS

Treatment	Total plasma bilirubin (mg/100 ml)
Control	0.2
Isopropanol	0.3
ANIT, 100 mg/kg	1.0*
Isopropanol + ANIT, 100 mg/kg	1.7*
ANIT, 300 mg/kg	2.6*
Isopropanol + ANIT, 300 mg/kg	4.2†

Isopropanol (2.5 ml/kg, p.o.) was given 18 hours before ANIT (100 or 300 mg/kg, p.o.). Liver function was assessed 24 hours after ANIT. (n=6).

*Significantly different from control, $p < 0.05$.

†Significantly different from group given ANIT alone, $p < 0.05$.

A last set of experiments was performed using ANIT. This substance does not produce necrosis, but causes a prompt cessation of bile flow (cholestasis) which results in hyperbilirubinemia within 24 hours. When rats were pretreated with isopropanol (2.5 ml/kg, p.o.), the hyperbilirubinemic response to a high dose of ANIT (300 mg/kg, p.o.) was significantly enhanced, although the response to a threshold dose (100 mg/kg, p.o.) was not affected (Table XVII). A similar experiment was performed using acetone (1.0 ml/kg, p.o.) pretreatment with similar results (Table XVIII). These preliminary results must be interpreted with caution because the extrahepatic effects of isopropanol and acetone on bilirubin disposition have not been studied. However, they suggest that isopropanol and acetone might affect non-necrogenic hepatic alterations as well. A similarity does exist between ANIT and CCl_4 in that CCl_4 -induced necrosis, as well as ANIT-induced cholestasis and hyperbilirubinemia, can be enhanced by pretreatment with phenobarbital (Roberts and Plaa, 1965; Garner and McLean, 1969.) However, in this situation, it is the threshold doses of ANIT whose effects are enhanced. This difference between isopropanol and phenobarbital is quite striking and more work is needed to fully evaluate its significance.

TABLE XVIII

EFFECT OF ACETONE PRETREATMENT ON ANIT-INDUCED HYPERBILIRUBINEMIA
IN RATS

Treatment	Total plasma bilirubin (mg/100 ml)
Control	0.4
Acetone	0.3
ANIT, 100 mg/kg	1.3*
Acetone + ANIT, 100 mg/kg	1.7*
ANIT, 300 mg/kg	1.5*
Acetone + ANIT, 300 mg/kg	3.1†

Acetone (1.0 ml/kg, p.o.) was given 18 hours before ANIT (100 or 300 mg/kg, p.o.). Liver function was assessed 24 hours after ANIT. (n=6).

*Significantly different from control, $p < 0.05$.

†Significantly different from group given ANIT alone, $p < 0.05$.

SUMMARY

Pretreatment of mice and rats with ethanol or isopropanol has been shown to enhance the hepatotoxic response of these animals to CCl_4 . The hepatotoxic effects of CCl_4 were assessed 24 hours after its administration by measuring SGPT activity, accumulation of liver triglycerides and depression of hepatic glucose-6-phosphatase activity. Maximal enhancement of CCl_4 hepatotoxicity was observed when the alcohol was given 18 hours before the CCl_4 . Isopropanol exerted a much greater potentiating effect than ethanol.

The ethanol- CCl_4 interaction seems to be due to an effect exerted by unchanged ethanol. However, with isopropanol it seems that both unchanged isopropanol and its metabolic product, acetone, are responsible for the enhanced response to CCl_4 ; acetone when given alone also potentiates CCl_4 hepatotoxicity. Although the mechanisms involved in the interaction are unknown, it seems that the metabolic activity of enzymes associated with the endoplasmic reticulum is involved.

Isopropanol and acetone pretreatment in mice also results in the enhancement of the hepatotoxic effects of chloroform, trichloroethylene and 1, 1, 2-trichloroethane; the effects of 1, 1, 1-trichloroethane, however, are not enhanced. No evidence was found to indicate that necrogenic responses to beryllium, allyl alcohol and galactosamine were enhanced in rats pretreated with isopropanol. Preliminary data suggest that the hyperbilirubinemia due to α -naphthylisothiocyanate treatment is also enhanced by both isopropanol or acetone pretreatment.

The isopropanol enhancing effect appears to be relatively specific. Only those agents which act predominantly on centrilobular hepatic cells were the ones found to be potentiated. This further supports the hypothesis that somehow the microsomal activity of the endoplasmic reticulum is involved in the interaction.

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Pyrazole Inhibition of Ethanol, Allyl Alcohol and Allyl Formate-Induced Hepatic Injury

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INTRODUCTION

Previous studies from this laboratory have contributed to the formulation of the hypothesis that the mechanism of hepatic cell injury after the administration of a variety of hepatotoxic agents is the result of enhanced lipid peroxidation (for review see Di Luzio, 1973). In general, this concept, proposed approximately 10 years ago, has found confirmation in a variety of laboratories (Takada, Ikegami, Okumura, Hasumura, Kanayama and Takeuchi, 1970; Comporti, Burdino and Raja, 1971; Recknagel, 1967; and Porta, Hartroft and de La Iglesia, 1967). While controversy has developed as to the role of lipid peroxidation relative to the ethanol-induced fatty liver (Hashimoto and Recknagel, 1967; Scheig and Klatskin, 1969) as well as the carbon tetrachloride-induced injury (Green, Bunyan, Cawthorne and Diplock, 1969) and the matter is yet to be resolved, the protective effect of antioxidants on certain types of chemical-induced injury has been fairly well established.

The lipid peroxidation hypothesis implies that hepatic injury results not from the direct effects of the agent per se but due to its metabolites. An attempt to demonstrate the validity of this concept employing pyrazole, an agent which inhibits alcohol dehydrogenase (Theorell, Yonetani and Sjöberg, 1969; Li and Theorell, 1969; Goldberg and Rydberg, 1969; and Lester, Keokosky and Felzenberg, 1968), demonstrated that fatty liver does not develop following the administration of pyrazole and depression of ethanol oxidation (Morgan and Di Luzio, 1970). These findings demonstrated that ethanol, per se, is not the causative factor in fatty liver development, stressing the possible toxic effects of acetaldehyde or alterations in the redox state of the liver cell.

The inhibitory effects of pyrazole or 4-methylpyrazole on acute ethanol-induced fatty liver have been, in general, demonstrated in a variety of laboratories (Blomstrand and Forsell, 1971; Domanski, Rifenberick, Stearns, Scorpio and Narrod, 1971; and Johnson, Hemell, Fex and Olivecrona, 1971) although Bustos, Kalant, Khanna and Loth (1970) were unable to demonstrate protection of the acute ethanol-induced fatty liver following pyrazole administration.

In an effort to extend the concept that toxic metabolites rather than the original parent compounds themselves are the hepatotoxic agents and that a key enzyme involved in the conversion of parent compounds into toxic metabolites is alcohol dehydrogenase, additional studies were undertaken employing two additional hepatotoxic agents which, like ethanol, require alcohol dehydrogenase for their metabolism. Allyl formate produces extensive periportal necrosis and hepatic cellular dysfunction (Piazza, 1915 and Rees and Tarlow, 1967) as does its metabolite, allyl alcohol (Piazza, 1915; Infante, Schwarzmann, Petit, Raisonnier and Caroli, 1969; Rees and Tarlow, 1967; and Reid, 1972). Allyl alcohol, when metabolized, produces acrolein, (Rees and Tarlow, 1967; Reid, 1972; and Serafini-Cessi, 1972) which also has the ability to produce hepatic necrosis.

In an effort to extend the concept of selective inhibition of alcohol dehydrogenase as a mechanism to modify hepatotoxicity induced by certain chemicals, hepatic parenchymal and Kupffer cell function, as well as determinations of liver triglyceride concentrations, were undertaken in allyl alcohol and allyl formate-treated animals. Additionally, light and electron microscopic studies were conducted to determine changes in the ultrastructure of liver cells in the presence of pyrazole and allyl formate. Additional studies employing pyrazole and disulfiram were also undertaken in reference to the role of metabolites in the induction of the acute ethanol-induced fatty liver.

Leibach (1969) has reported prolonged administration of ethanol together with pyrazole-produced hepatic necrosis and death in rats. Since Lieber, Rubin, DeCarli, Misra and Gang (1970) have reported that pyrazole produces alterations in hepatic function and morphology, studies were undertaken to evaluate the influence of pyrazole administration on plasma retention of bromsulfalein (BSP), plasma bilirubin and Kupffer cell phagocytosis. Additionally, since the protective effect of pyrazole, in modifying the ethanol-induced fatty liver, may involve its action on other metabolic substrates, the comparative influence of pyrazole on the *in vivo* metabolism of ethanol, acetate, acetaldehyde, glucose and palmitic acid was ascertained.

METHODS

In studies on the ability of pyrazole and Antabuse to modify the development of the acute ethanol-induced fatty liver, male Sprague-Dawley rats received pyrazole (50 mg/100 g) intraperitoneally. Saline was injected in equivalent volumes in control animals. Antabuse was administered by oral intubation in the amount of 60 mg/100 g as a saline suspension. Sixteen hours later, ethanol (6 g/kg) or saline was administered by oral intubation. Liver triglyceride concentration was ascertained sixteen hours later.

In studies on the influence of pyrazole administration on allyl alcohol or allyl formate induced hepatic injury, male rats received pyrazole (36 mg/100 g) or saline by intraperitoneal administration. Four hours following the administration of either saline or pyrazole, allyl alcohol was administered intraperitoneally in the amount of 5 mg/100 g of body weight. Twenty hours later, liver triglycerides were determined by the method of Van Handle and Zilversmit (1957). Plasma BSP retention was measured at this time

following the administration of 5 mg BSP/100 g body weight. The plasma concentration of BSP was determined 30 minutes following injection.

Allyl formate was administered intraperitoneally in the amount of 7.1 mg/100 g body weight four hours following the administration of saline or pyrazole. Twenty hours following the administration of allyl formate, BSP was injected in the amount of 5 mg/100 g body weight for the determination of liver function. Plasma bilirubin was also measured at this time as well as liver triglyceride concentrations. Liver sections were obtained for light and electron microscopic studies in various groups.

Similar studies were conducted on the ability of pyrazole to modify allyl formate and allyl alcohol induced lethality. A similar design was employed in which pyrazole or saline was administered four hours prior to administration of the hepatotoxin. In this study, allyl formate or allyl alcohol was administered intraperitoneally in the amount of 7.1 and 6.3 mg/100 g respectively and mortality ascertained.

The metabolism of ^{14}C -labeled substrates to $^{14}\text{CO}_2$ was determined by placing individual rats in glass metabolism cages (Delmar Scientific Labs, Maywood, Illinois). In this instance, female rats were injected with pyrazole (35 mg/100 g) intraperitoneally. Control rats received saline injections. One hour later, the rats received an intraperitoneal injection of 1 to 6 μC of one of the following ^{14}C -labeled preparations: ethanol, acetate, acetaldehyde, and palmitic acid prepared in rat plasma. ^{14}C -(u) glucose was administered by oral intubation. The temperature of the metabolism cage was 25°C and CO_2 free air flow in the system approximated 1 liter per minute. The expired CO_2 was trapped in a solution of ethylene glycol monomethyl ether (methyl cellosolve) and 2-amino-ethanol (2:1 v/v). At appropriate intervals, the CO_2 trapping solutions were removed and radioactivity ascertained in a mixture of toluene, methyl cellosolve and 2, 5-diphenyloxazole according to the procedure of Jeffay and Alvarez (1961).

The intravascular clearance and tissue distribution of the ^{131}I -triolein labeled reticuloendothelial test lipid emulsion were ascertained by previously described procedures (Pisano, Patterson, and Di Luzio, 1969) in allyl formate (7.1 mg/100 g) treated rats in the presence and absence of pyrazole administration. Pyrazole or saline was administered four hours prior to allyl formate administration. Reticuloendothelial function, as reflected by the intravascular clearance and tissue distribution of the emulsion, was ascertained twenty hours after allyl formate administration.

RESULTS

In agreement with all previous observations, the administration of ethyl alcohol to rats produced a significant increase in liver triglyceride concentration. In agreement with previous observations (Morgan and Di Luzio, 1970), the administration of pyrazole alone did not modify liver triglyceride levels. The prior administration of pyrazole was effective in completely preventing the ethanol-induced fatty liver (Table I). Similar studies conducted at 6 hours following ethanol administration also indicated an inhibitory effect of pyrazole on the ethanol-induced increment in liver triglyceride content.

Antabuse (disulfiram) was also employed to impair aldehyde dehydrogenase and to produce an accumulation of acetaldehyde in an attempt to denote whether fatty liver would occur under such conditions. Antabuse administered alone produced a significant increase in liver triglycerides (Table I). The administration of ethanol (6 g/kg) to disulfiram-treated rats did not result in any increment in liver triglyceride concentration,

TABLE I

PROTECTIVE EFFECT OF PYRAZOLE AND ANTABUSE ON
ACUTE ETHANOL-INDUCED FATTY LIVER

Group	Treatment Pyrazole	Antabuse	Number of Animals	Liver Triglycerides mg/g
Saline	-	-	11	2.6 ± 0.28
Ethanol	-	-	12	7.4 ± 0.99
Saline	+	-	9	1.6 ± 0.22
Ethanol	+	-	8	1.4 ± 0.35
Saline	-	+	9	7.8 ± 1.21
Ethanol	-	+	9	7.4 ± 0.71

Liver triglyceride concentrations were determined in male rats 16 hours after gastric lavage of ethanol (6 g/kg). Animals were pretreated 16 hours prior to ethanol administration with either pyrazole (50 mg/100 g), Antabuse (60 mg/100 g) or saline. All liver samples were analyzed in triplicate. Liver triglyceride values are expressed as mean ± standard error.

suggesting that acetaldehyde may not be the metabolite which induces fatty liver development.

The influence of pyrazole on allyl alcohol-induced mortality is presented in Table II. The administration of allyl alcohol in a dose of 6.3 mg/100 g resulted in a 100% mortality. The prior administration of pyrazole completely prevented the allyl alcohol induced mortality.

In agreement with the ability of pyrazole to modify allyl alcohol induced mortality, no deaths occurred in the allyl formate group which received a prior injection of pyrazole (Table III). Thus, pyrazole is capable of modifying allyl formate and allyl alcohol induced mortality.

TABLE II

PROTECTIVE INFLUENCE OF PYRAZOLE ON
ALLYL ALCOHOL INDUCED MORTALITY

Treatment	Mortality %
Saline & Saline	0
Saline & Allyl alcohol	100
Pyrazole & Saline	0
Pyrazole & Allyl alcohol	0

Allyl alcohol was administered intraperitoneally in the dose of 6.35 mg/100 g four hours after the administration of either pyrazole (35 mg/100 g) or saline. Each group consisted of six rats. Mortality ascertained 48 hours following allyl alcohol administration.

The administration of allyl formate induced severe liver dysfunction which was reflected by an approximate 12-fold increase in plasma retention of BSP (Table IV). Likewise, bilirubin concentration was significantly increased (Table IV). Pyrazole-treated animals did not display any modification in plasma BSP concentration; however, a slight but significant increase in plasma bilirubin concentration was observed.

TABLE III

PROTECTIVE INFLUENCE OF PYRAZOLE ADMINISTRATION
ON ALLYL FORMATE INDUCED MORTALITY

Treatment	Deaths/No.	Mortality %
Saline & Saline	0/20	0
Saline & Allyl formate	15/30	50
Pyrazole & Saline	0/20	0
Pyrazole & Allyl formate	0/20	0

Allyl formate was administered intraperitoneally in the dose of 7.1 mg/100 g four hours after the administration of either pyrazole or saline. Percent mortality ascertained 48 hours post-treatment.

TABLE IV

INFLUENCE OF PYRAZOLE ADMINISTRATION ON PLASMA
BILIRUBIN AND BROMSULFALEIN RETENTION FOLLOWING
ALLYL FORMATE ADMINISTRATION

Treatment	No.	Plasma BSP mg%	Plasma total Bilirubin mg%
Saline & Saline	10	1.54 \pm 0.30	0.22 \pm 0.03
Saline & Allyl formate	10	13.5 \pm 2.14	0.73 \pm 0.12
Pyrazole & Saline	10	1.42 \pm 0.17	0.39 \pm 0.11
Pyrazole & Allyl formate	10	2.67 \pm 0.61	0.44 \pm 0.10

Values are expressed as means \pm standard error. Allyl formate (7.1 mg/100 g) was administered intraperitoneally 4 hours following pyrazole administration (36 mg/100 g). Plasma bilirubin and bromsulfalein were determined 20 hours following allyl formate or saline administration.

In contrast to the profound elevation in plasma BSP retention noted in allyl formate-treated animals, the allyl formate group which received pyrazole manifested normal plasma BSP levels. Likewise, the allyl formate-induced increase in plasma bilirubin concentration was not observed in the allyl formate-treated animals that received pyrazole.

Allyl formate administration was also associated with a significant increase in liver weight, liver triglyceride concentration and liver triglyceride content (Table V). In agreement with the ability of pyrazole to modify allyl formate-induced alterations in plasma

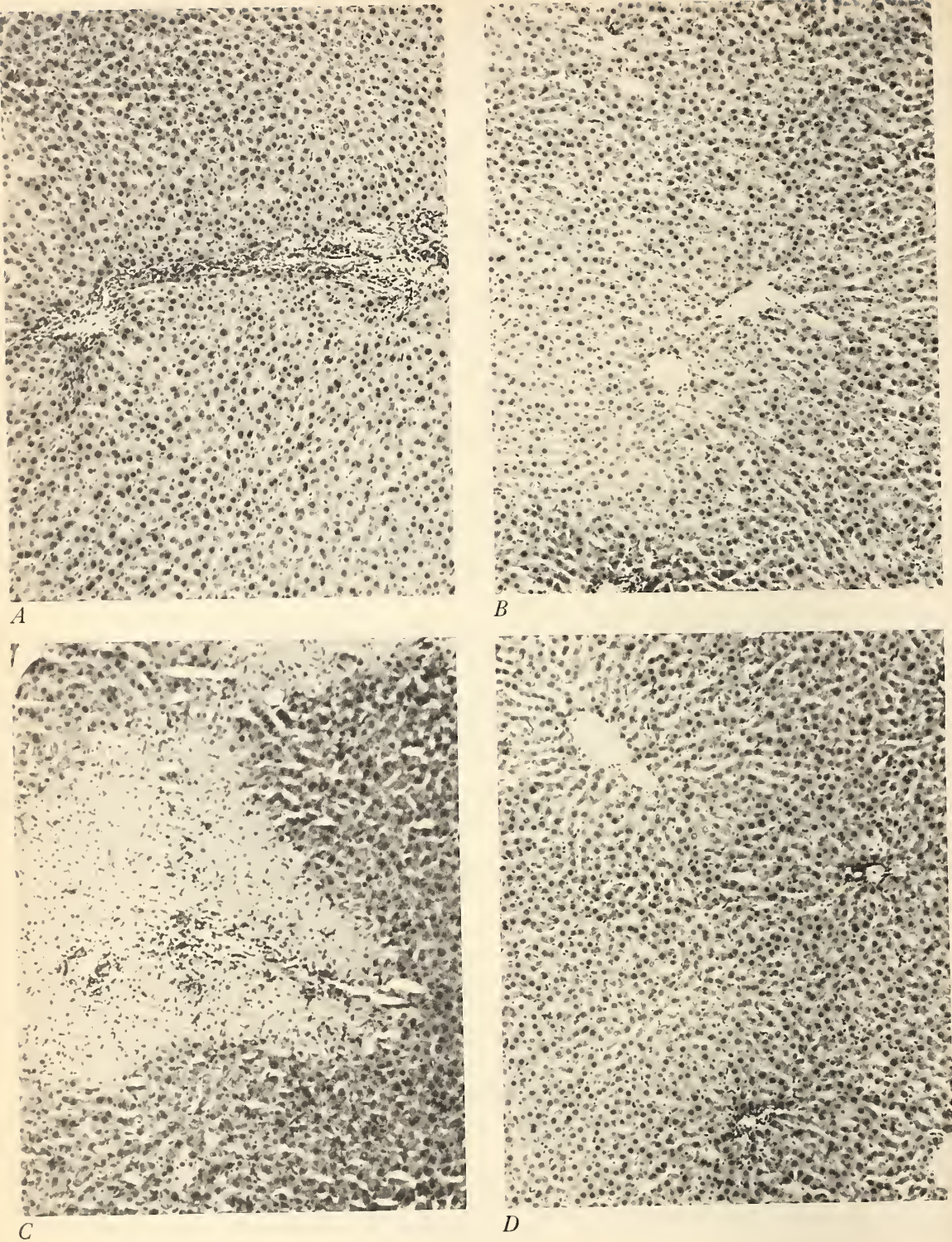


Figure 1. *Light microscopic sections from rats treated with either saline (A), pyrazole (B), allyl formate (C), and pyrazole + allyl formate (D). The profound periportal necrosis is clearly evident in the allyl formate-treated liver as is the inhibition of necrosis in pyrazole-allyl formate-treated rats.*

TABLE V

INFLUENCE OF PYRAZOLE ADMINISTRATION ON LIVER WEIGHT
AND TRIGLYCERIDE CONTENT FOLLOWING ALLYL FORMATE ADMINISTRATION

Treatment	No.	Liver wgt. (g)	Liver Triglyceride	
			mg/g	mg/liver
Saline & Saline	13	7.0 \pm 0.42	3.0 \pm 0.26	21.4 \pm 2.2
Saline & Allyl Formate	12	10.0 \pm 0.35	8.0 \pm 1.08	75.6 \pm 12.3
Pyrazole & Saline	13	8.3 \pm 0.51	2.8 \pm 0.48	18.7 \pm 2.7
Pyrazole & Allyl Formate	13	8.7 \pm 0.60	3.1 \pm 0.44	28.1 \pm 4.4

Values are expressed as means \pm standard error. Liver triglycerides were determined 20 hours following allyl formate (7.1 mg/100 g) or saline. Pyrazole or saline was administered intraperitoneally 4 hours prior to allyl formate administration.

BSP concentrations and total bilirubin, the rats that received pyrazole and allyl formate showed normal triglyceride concentrations and liver weight. Thus, by these assessments, hepatic integrity is maintained following the administration of allyl formate if pyrazole is administered to block alcohol dehydrogenase and thus the metabolism of allyl formate.

In agreement with previous findings, light microscopic studies following allyl formate administration revealed extensive cell necrosis about the periportal space (Fig. 1). A significant infiltration of inflammatory cells was also manifested in this area. The integrity of the parenchymal cell was disrupted, degenerative changes and necrosis were prominent. Staining the sections with lipid stains revealed a significant increase in fat droplets. Administration of pyrazole alone was associated with no demonstrable light microscopic changes. The allyl formate-treated group which previously received pyrazole showed hepatic architecture which was very similar to the control livers (Fig. 1). A slight increase in lipid droplets appears to be manifested in this group compared to control livers.

Electron microscopic observations of liver in the allyl formate-treated group, when compared to control livers (Fig. 2) revealed marked degenerative changes in hepatic parenchymal cells which were characterized by intense mitochondrial distension, disappearance of their cristae and increase in size of dense bodies (Fig. 3). The parenchymal cell membranes were frequently found to be ruptured. There was a marked dilatation in the smooth endoplasmic reticulum and a partial disappearance of the rough endoplasmic reticulum in the allyl formate-treated rats. A marked increase in smooth membrane vesicles was also observed as was enhancement in the number of lipid droplets. The nucleus of the cell showed vesiculation, aggregation of the chromatin and dilatation of the nuclear envelope. An increase in polymorphonuclear leukocytes was observed in the sinusoids. The ultrastructural changes were not limited exclusively to parenchymal cells, as Kupffer cells displayed a marked increase in lipid droplets, degenerative changes, and necrosis. In the rats which received pyrazole alone, there appeared to be an increase in dense bodies of the mitochondria, increase and dilatation of smooth endoplasmic reticulum and slight tendency for lipid vacuolization. Parenchymal cells and Kupffer cells, in general, had slight ultrastructural changes (Fig. 4).

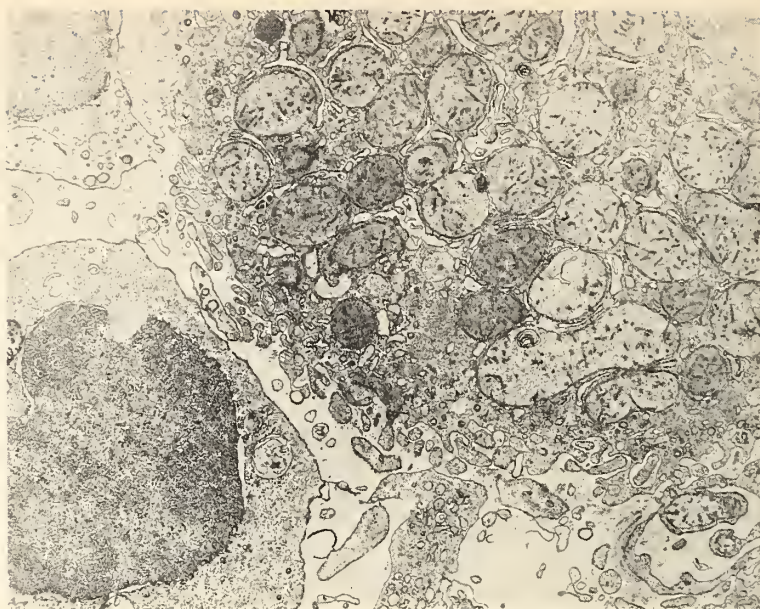


Figure 2. *Electron micrographic section of the liver from a rat following saline administration. Notice the integrity of mitochondria, the well delineated space of Disse and the normal amount of smooth endoplasmic reticulum. A lymphocyte (lower left) and a Kupffer cell (upper left) are present in the sinusoid. 8000X.*

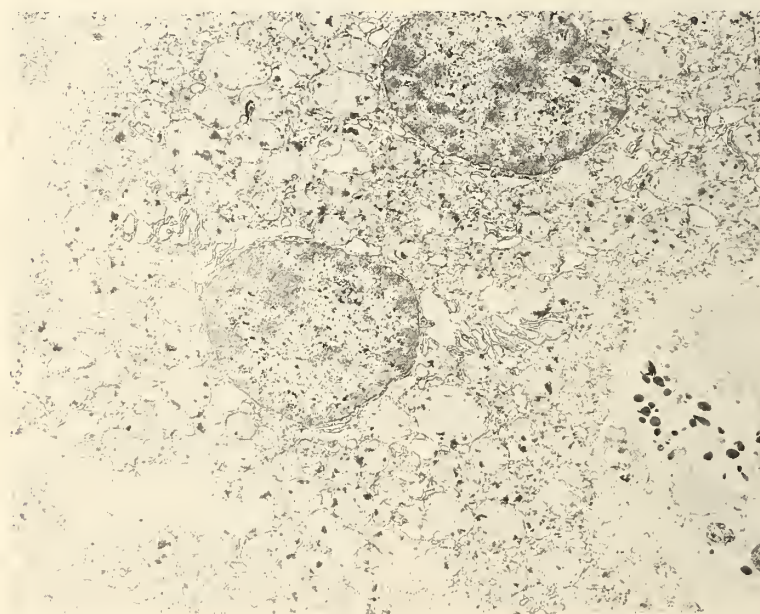


Figure 3. *Electron micrographic section of liver parenchymal cell from rat 20 hours following allyl formate administration shows pronounced hepatic cellular disruption, fat droplets, loss of mitochondrial cristae with deposits of an electron dense material. Dilatation of the endoplasmic reticulum, loss of cellular membrane, and aggregated nuclear chromatin can also be observed. A polymorphonuclear neutrophil is present in the sinusoid. 6000X.*

In the allyl formate-treated group which received a prior injection of pyrazole, parenchymal cells showed slight changes similar to those described after pyrazole (Fig. 5). However, in general, a normal ultrastructural appearance was manifested. Lipid droplets appeared a bit more frequently in parenchymal cells of the allyl formate-pyrazole group. Kupffer cells appeared vacuolated but otherwise were essentially normal.

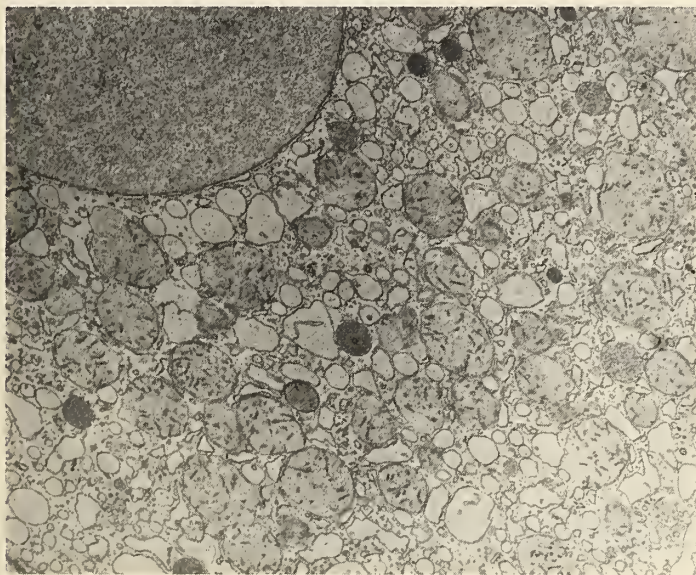


Figure 4. *Ultrastructure of the liver parenchymal cells from a rat after pyrazole injection. There is slight deformity in mitochondrial shape, dense granules appear more prominent and there is a modification and slight dilatation of the smooth endoplasmic reticulum. 6000X.*

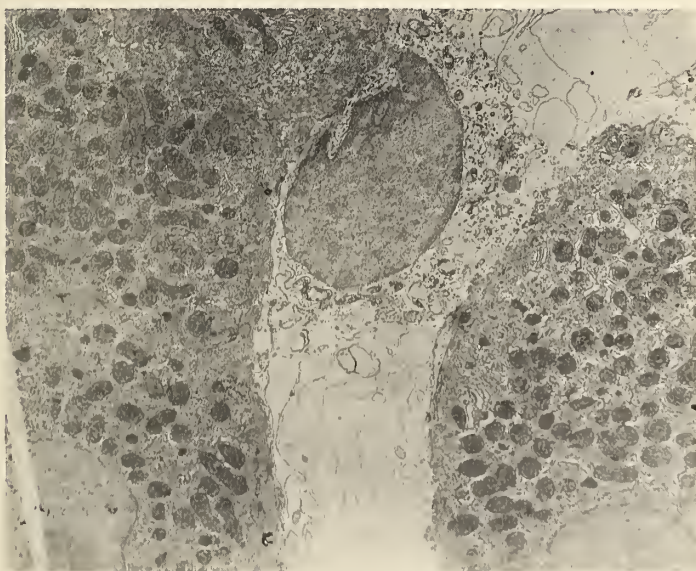


Figure 5. *Electron micrographic section of liver 20 hours following allyl formate administration in a rat which was previously treated with pyrazole four hours before allyl formate administration. Compared to Fig. 3, the profound reduction in allyl formate-induced cellular degenerative changes by pyrazole is apparent. Vacuolization and disruption of a Kupffer cell is also evident.*

In an effort to ascertain whether antioxidants which have demonstrated ability to modify various types of chemical-induced hepatic injury could also modify allyl formate-induced injury, studies were undertaken in which DPPD (60 mg/100 g) was administered intraperitoneally as a suspension of corn oil 48 and 24 hours prior to the administration of allyl formate. The administration of allyl formate produced an approximate 9-fold increase in the plasma retention of BSP (Table VI). Administration of DPPD alone did not alter plasma BSP retention. In contrast to the protective effect of pyrazole, DPPD administration did not prevent the significant retention of plasma BSP induced by allyl formate.

TABLE VI
FAILURE OF DPPD ADMINISTRATION TO MODIFY
ALLYL FORMATE INDUCED IMPAIRMENT OF BSP UPTAKE

Treatment	No.	Plasma BSP mg%
Corn oil & Saline	13	1.6 ± 0.29
Corn oil & Allyl formate	7	13.6 ± 4.0
DPPD & Saline	12	1.4 ± 0.19
DPPD & Allyl formate	8	9.1 ± 2.5

N,N¹-diphenyl-p-phenylenediamine (DPPD) was administered intraperitoneally in the amount of 60 mg/100 g as a corn oil suspension 48 and 24 hours prior to administration of allyl formate (7.1 mg/100 g).

Since allyl alcohol is a metabolite of allyl formate, studies were undertaken to determine whether allyl alcohol itself is hepatotoxic or whether it is converted to a toxic metabolite. The administration of allyl alcohol produced an approximate 11-fold increase in plasma BSP concentration (Table VII). In accordance with previous findings, pyrazole alone did not modify plasma BSP levels. In agreement with the observations with allyl formate, the prior administration of pyrazole to animals which received allyl alcohol was associated with normal plasma BSP concentrations. Thus, pretreatment with pyrazole was capable of modifying the retention of BSP induced by allyl alcohol administration.

Studies were also undertaken to determine whether allyl alcohol produced changes in liver triglyceride concentration which were modifiable by pyrazole administration. In contrast to the influence of allyl formate, allyl alcohol alone did not significantly modify liver triglycerides (Table VIII). Thus, it was impossible to determine whether any change would be observed in the pyrazole-treated animal.

Previous observations stress parenchymal cell activity and the influence of allyl formate and allyl alcohol thereupon. In an effort to ascertain whether functional changes occur in hepatic Kupffer cells which were associated with marked ultrastructural observations following administration of allyl formate, the intravascular clearance and organ uptake of the gelatinized reticuloendothelial test lipid emulsion was determined in control and allyl formate-treated animals in the presence and absence of pyrazole.

TABLE VII

PROTECTIVE EFFECT OF PYRAZOLE ON ALLYL ALCOHOL
INDUCED BSP RETENTION

Treatment	No.	Plasma BSP mg%
Saline & Saline	4	0.89 ± 0.18
Saline & Allyl Alcohol	12	10.4 ± 2.37
Pyrazole & Saline	5	1.20 ± 0.35
Pyrazole & Allyl Alcohol	15	1.43 ± 0.25

Allyl alcohol was administered in the amount of 5 mg/100g four hours following the administration of pyrazole (36 mg/100g) or saline. Plasma BSP determined 30 minutes following the intravenous administration of BSP (5 mg/100 g). The studies on retention of BSP were conducted 20 hours following allyl formate administration.

TABLE VIII

LIVER TRIGLYCERIDE CONCENTRATION FOLLOWING
ALLYL ALCOHOL AND PYRAZOLE

Treatment	No.	Liver wgt. g	Liver Triglyceride mg/g	mg/liver
Saline & Saline	4	11.1 ± 1.1	2.9 ± 0.8	31.1 ± 8.2
Saline & Allyl Alcohol	12	11.7 ± 0.6	4.3 ± 0.7	52.1 ± 10.1
Pyrazole & Saline	5	10.9 ± 0.9	2.2 ± 0.4	24.8 ± 5.8
Pyrazole & Allyl Alcohol	15	9.9 ± 0.3	3.6 ± 0.4	35.4 ± 3.6

Values are expressed as means ± standard error. Experimental details are outlined in Table VII.

Administration of allyl formate produced significant impairment in phagocytosis as reflected by a three-fold prolongation in the intravascular clearance of the reticuloendothelial test lipid emulsion (Table IX). Pyrazole itself exerted a slight but nonsignificant effect in inhibiting the vascular clearance of the labeled lipid emulsion. The administration of pyrazole prior to allyl formate administration did not significantly modify the impaired intravascular clearance rate.

The organ distribution of the lipid emulsion, which is predominantly hepatic in nature, was significantly depressed in allyl formate-treated animals (Table IX). The 50 per cent reduction in Kupffer cell phagocytosis observed following the administration of allyl formate was not significantly modified by prior pyrazole administration. These findings indicate that pyrazole is ineffective in modifying Kupffer cell dysfunction induced by allyl formate. Organ uptake by lung and spleen in pyrazole or allyl formate-treated animals did not differ significantly from normal values.

TABLE IX

IMPAIRMENT IN VASCULAR CLEARANCE AND KUPFFER CELL PHAGOCYTOSIS OF THE
GELATINIZED RETICULOENDOTHELIAL (RE) TEST LIPID EMULSION
FOLLOWING ALLYL FORMATE ADMINISTRATION

Group	No.	Intravascular $t/2$ min.	Organ Uptake, % ID		
			Liver	Lung	Spleen
Saline vs Saline	11	11.0 \pm 2.0	56.7 \pm 3.2	2.2 \pm 0.5	4.3 \pm 0.3
Pyrazole vs Saline	9	17.0 \pm 2.3	51.6 \pm 4.4	2.9 \pm 0.5	3.0 \pm 0.2
Saline vs Allyl Formate	10	33.9 \pm 6.4	23.4 \pm 4.1	2.2 \pm 0.3	3.5 \pm 0.6
Pyrazole vs Allyl Formate	9	28.8 \pm 5.2	37.6 \pm 5.6	2.9 \pm 0.5	2.6 \pm 0.2

Values are expressed as mean \pm standard error. The clearance and tissue distribution of the ^{131}I -triolein labeled RE test lipid emulsion was ascertained in allyl formate-treated rats which received either saline or pyrazole. RE function was ascertained 20 hours after allyl formate administration.

%ID = per cent of injected radioactivity.

TABLE X

INFLUENCE OF PYRAZOLE ON METABOLISM OF ETHANOL, ACETALDEHYDE,
ACETATE, GLUCOSE OR PALMITIC ACID^a

Labeled Compound	Treatment	$^{14}\text{CO}_2$ output, percent of injected dose			
		1 hr	3 hr	6 hr	24 hr
Ethanol	Pyrazole	2.7 \pm 0.4	10.0 \pm 1.4	19.8 \pm 2.0	50.5 \pm 6.6
Ethanol	Saline	5.4 \pm 0.7 ^b	21.7 \pm 3.2 ^b	51.6 \pm 5.3 ^c	75.9 \pm 4.9 ^b
Acetaldehyde	Pyrazole	26.5 \pm 1.7	53.7 \pm 1.6	61.5 \pm 1.7	68.1 \pm 1.8
Acetaldehyde	Saline	35.4 \pm 3.0 ^d	55.6 \pm 2.0	61.2 \pm 2.2	65.4 \pm 1.9
Acetate	Pyrazole	33.0 \pm 2.2	53.3 \pm 3.5	58.0 \pm 3.8	62.7 \pm 4.3
Acetate	Saline	32.3 \pm 2.6	54.0 \pm 1.3	59.5 \pm 1.6	63.6 \pm 1.9
Glucose	Pyrazole	5.1 \pm 1.0	25.6 \pm 3.1	41.5 \pm 2.5	52.4 \pm 1.4
Glucose	Saline	9.1 \pm 1.1 ^d	32.1 \pm 2.7	45.7 \pm 2.8	54.0 \pm 1.5
Palmitic Acid	Pyrazole	9.7 \pm 1.1	25.1 \pm 0.7	30.7 \pm 1.3	40.6 \pm 1.9
Palmitic Acid	Saline	9.2 \pm 2.1	17.9 \pm 3.5	21.2 \pm 4.0	31.0 \pm 4.2

^aValues of $^{14}\text{CO}_2$ output are expressed as means \pm standard error and are derived from 6-9 rats per group. Acetate and acetaldehyde were administered in the amount of 8 mg/100 g; ethanol — 30 mg/100 g; glucose — 50 mg/100 g and palmitic acid — 1.0 mg/100 g. Difference from control value at respective time period by Student's t test.

^b_p < 0.01 ^c_p < 0.001 ^d_p < 0.02

Since pyrazole may alter fundamental metabolic events in carbohydrate and lipid metabolism and thus inhibit ethanol-induced fatty liver development, the influence of pyrazole administration on the excretion of $^{14}\text{CO}_2$ from various labeled substrates was ascertained *in vivo*. In agreement with previous studies (Goldberg and Rydberg, 1969), pyrazole administration inhibited, but did not totally block, the *in vivo* oxidation of ethanol (Table X). The $^{14}\text{CO}_2$ output from labeled ethanol was significantly depressed at all evaluated periods in the pyrazole-treated rats. In contrast, $^{14}\text{CO}_2$ excretion from either 1-2- ^{14}C acetaldehyde, 1- ^{14}C acetate or 1- ^{14}C -palmitic acid was essentially unaltered. A significant depression of $^{14}\text{CO}_2$ output from ^{14}C -(u)-glucose was only observed at the one hour period, with normal value of $^{14}\text{CO}_2$ excretion occurring at later intervals.

DISCUSSION

As previously reported (Piazza, 1915; Schwarzmunn *et al.*, 1967; Rees and Tarlow, 1967; Infante *et al.*, 1969; Reid, 1972) allyl formate and allyl alcohol have been demonstrated to be significant hepatotoxic agents. The observations of Rees and Tarlow (1967) that the inhibition induced in mitochondria respiration and depression of protein synthesis by allyl formate depends upon the activity of alcohol dehydrogenase is essentially supported in the present observation. Indeed, the pronounced periportal necrosis which is seen following the administration of allyl formate and allyl alcohol (Rees and Tarlow, 1967; Reid, 1972; Infante *et al.*, 1969) has been postulated to reflect the distribution of alcohol dehydrogenase (Rees and Tarlow, 1967) which is localized primarily in the periportal regions of the hepatic lobule. Present findings of pyrazole inhibition of alcohol dehydrogenase and resulting decrease in hepatic injury also supports the postulate of Rees and Tarlow (1967) that the periportal distribution of allyl formate-induced hepatic necrosis is due to alcohol dehydrogenase localized in that area.

Infante *et al.* (1969) have reported hepatic lesions and liver dysfunction as reflected by the passage of cytoplasmic enzymes into the perfusion medium following the administration of allyl alcohol to isolated perfused liver systems. This group (Schwarzmunn *et al.*, 1967) has also reported the prevention of allyl alcohol-induced hepatic lesions by ethyl alcohol administration. These studies suggest that the preferential oxidation of ethyl alcohol by alcohol dehydrogenase, resulting in impairment in metabolism of allyl alcohol, is capable of reducing allyl alcohol-induced hepatic injury. The present findings are in accord with these concepts.

Rees and Tarlow (1967) have also reported that glutathione and 1, 4-dithiothreitol protected against early biochemical alterations produced by allyl formate administration. Rees and Tarlow (1967) also suggested that allyl formate is not the toxic agent but that it is converted to allyl alcohol and then to acrolein, a reaction which requires the presence of alcohol dehydrogenase. Since acrolein is a high reactive aldehyde which could react with various components of the cell, thiol compounds were administered to modify possible reactions of acrolein with cell components (Rees and Tarlow, 1967). Protection of allyl alcohol-induced hepatic injury was mediated by glutathione and 1, 4-dithiothreitol. Since both of these compounds have antioxidant properties, it was somewhat surprising that DPPD, which is effective in modifying a variety of chemical induced types of hepatic dysfunction (Di Luzio, 1973) did not modify the allyl formate induced hepatic dysfunction as reflected by BSP uptake. The lack of effectiveness of DPPD, in contrast to

the effectiveness of 1, 4-dithiothreitol and glutathione may be due to the water insoluble nature of DPPD, precluding any effective action at aqueous sites.

The studies of the inhibition of alcohol, allyl alcohol and allyl formate-induced liver injury by blocking the metabolism of these compounds directly relate to studies with bromobenzene metabolism (Reid, Christie, Krishna, Mitchell, Moskowitz and Brodie, 1971) and carbon tetrachloride-induced injury (Recknagel, 1967). In both cases, the hepatotoxic effect of these agents is mediated by a metabolite rather than the parent compound itself.

Watkins, Goodman and Tephly (1970) reported that the administration of a single dose of pyrazole did not induce changes in serum glutamic oxalacetic transaminase levels or hepatic structure as denoted by light microscopy. While the present studies indicate that pyrazole itself in a single dose does not alter liver function as reflected by plasma clearance of BSP, a distinct increase in bilirubin concentration was observed. This latter finding is in agreement with the observations of Lieber *et al.* (1970) that a single injection of pyrazole will elevate plasma bilirubin concentration.

In essential agreement with the ability of pyrazole to modify the ethanol-induced increment in hepatic triglyceride concentration, pyrazole has been demonstrated by Krebs, Freedland, Hems and Stubbs (1969) to reverse the ethanol-induced inhibition of gluconeogenesis. Likewise, Estler (1972) reported that pyrazole completely prevented the ethanol-induced rise of hepatic glycerol-1-phosphate content and of the glycerol-1-phosphate/dihydroxyacetone phosphate and lactate/pyruvate ratios. Estler suggested that these changes are mediated by shifts in the hepatic NADH/NAD ratio during alcohol oxidation. However, since the glycogen depleting effect of ethanol was not abolished by pyrazole, Estler felt this effect is produced, at least in part, by ethanol itself.

Ontko (1973) employing isolated hepatic parenchymal cells, demonstrated ethanol inhibition of palmitate oxidation associated with an increased esterification of fatty acid into triglycerides and decreased formation of ketone bodies. Since these effects were abolished by pyrazole, he also concluded that ethanol oxidation was a requirement for the classical ethanol-induced changes in lipid metabolism. These composite studies clearly denote that classical ethanol-induced metabolic alterations are the result of either ethanol metabolites or associated redox potentials of the liver cell. The finding that disulfiram administration, which leads to the accumulation of acetaldehyde, prevented the ethanol-induced fatty liver appears to preclude acetaldehyde as the toxic agent. This finding stresses the possibility of the excessive and rapid accumulation of acetate (alone or in the presence of ethanol and/or acetaldehyde) or changes in the redox state of the cell as a factor in fatty liver development.

The present studies, which denote that various alcohol derivatives are themselves not hepatotoxic but require metabolic conversions, further stress the appreciation of understanding such alterations in the pathogenesis of chemical-induced hepatic injury. Through such information, it may well be possible in the future to significantly modify a variety of insults to the liver induced by various chemical or environmental agents.

SUMMARY

Employing a variety of hepatotoxic agents which require liver alcohol dehydrogenase for their metabolic conversions, studies were undertaken to evaluate whether the administered compound or its metabolites are involved in the induction of liver injury. Ethyl alcohol, allyl alcohol and allyl formate were administered to rats which previously

received either pyrazole or saline. Pyrazole, which effectively inhibits alcohol dehydrogenase prevented the acute ethanol-induced fatty liver. Antabuse, which impairs aldehyde dehydrogenase also inhibited fatty liver development. Allyl alcohol and allyl formate-induced lethality was completely prevented by pyrazole demonstrating the importance of metabolic conversion of these compounds to acrolein as a factor in their toxicity. Pyrazole also maintained hepatic parenchymal cell function as reflected by normalization of bromsulfalein retention in both allyl alcohol and allyl formate-treated rats. In allyl formate-treated rats the characteristic periportal necrosis was completely prevented by pyrazole. In contrast to the ability of pyrazole to protect the hepatic parenchymal cell from allyl formate-induced injury, depression of Kupffer cell phagocytosis by allyl formate was not modified by pyrazole. Pyrazole, while significantly inhibiting *in vivo* the $^{14}\text{CO}_2$ production from labeled ethanol, did not modify the $^{14}\text{CO}_2$ excretion from labeled glucose, acetate, acetaldehyde and fatty acid. These studies suggest that the inhibition of the ethanol-induced fatty liver by pyrazole is not related to the influence of pyrazole on other biochemical events.

The ability of pyrazole as well as disulfiram to inhibit the acute ethanol induced fatty liver focuses attention on the terminal metabolite of ethanol as well as changes in redox potential of cell induced by the metabolism of ethanol as a principal factor in the induction of hepatic cell injury.

ACKNOWLEDGEMENT

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Effects of Pyrazole on Acute and Chronic Ethanol-Induced Fatty Liver

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One of the prominent effects which can be observed after acute and chronic administration of alcohol in man and experimental animals, is the deposition of fat in the liver. To explain the pathogenesis of fatty liver, various hypotheses have been propounded (for references, see Hawkins and Kalant, 1972). One widely accepted view is that hepatic fat accumulation is primarily the result of disturbances in intermediary metabolism resulting from the oxidation of ethanol (Lieber, Rubin and DeCarli, 1971). The resulting intrahepatic accumulation of NADH_2 inhibits the oxidation of free fatty acids (Lieber and Schmidt, 1961; Rebouças and Isselbacher, 1961; Williamson, Scholz, Browning, Thurman and Fukami, 1969), favors the conversion of dihydroxyacetone phosphate to α -glycerophosphate, and thus increases esterification to form triglycerides (Nikkila and Ojala, 1963; Zakim, 1965).

Indirect evidence of several types, however, is not consistent with the view that the acute model of alcohol-induced fatty liver results primarily from these metabolic disturbances:

1. Small doses of ethanol do not cause triglyceride (TG) accumulation, although they are large enough to cause intrahepatic accumulation of NADH_2 (Kalant, Khanna and Loth, 1970; Maling, Highman, Hunter and Butler, 1967).

2. The degree of TG accumulation is related to the dose of ethanol administered, even though ethanol oxidation is a zero-order process at all but very low ethanol concentrations (Maling *et al.*, 1967).

3. Administration of pyrazole in a dose which completely prevented ethanol metabolism and ethanol-induced shift in nucleotides did not affect TG accumulation (Bustos, Kalant, Khanna and Loth, 1970).

4. In rats maintained on a diet deficient in protein, whereby ethanol oxidation and its consequences such as shift in nucleotides were strongly inhibited, the accumulation of TG in the liver after a single large dose of ethanol was even more pronounced than in control animals (Bode, Bode, Goebell, Kono and Martini, 1971).

In contrast, there is no doubt that the events leading to a fatty liver during chronic alcohol ingestion are closely associated with ethanol metabolism. However, the significance of metabolism in relation to the development of fatty liver remains to be defined fully, since an increase in NADH₂ content and inhibition of fatty acid oxidation have been observed in animals following the administration of such substances as sorbitol, xylitol, fructose or glucose, although none of them produce fat accumulation in the liver (Feigl, 1918; Rebouças and Isselbacher, 1961). Some newer work also suggests that ethanol *per se* may be hepatotoxic. Lelbach (1969) has reported structural changes resembling liver cell necrosis and cirrhosis in rats chronically treated with ethanol in the presence of pyrazole. He suggested that the hepatic changes may have been caused by the prolonged presence of ethanol itself. However, this work needs confirmation in view of the lack of adequate controls for the effect of pyrazole alone in these studies.

Since pyrazole has been shown to inhibit effectively the metabolism of ethanol both *in vivo* and *in vitro* (Theorell, 1965; Lester, Keokosky and Felzenberg, 1968; Goldberg and Rydberg, 1969; Bustos *et al*, 1970), it can be a very valuable tool for separating those effects of ethanol due to its metabolism from those due to ethanol *per se*. We have therefore investigated the effect of pyrazole on the accumulation of TG in the liver in animals receiving ethanol acutely and chronically, to test whether ethanol-induced fatty liver results from the metabolism of ethanol or from pharmacological actions of ethanol *per se*. The results of the studies to be presented suggest that under conditions of acute and chronic ethanol administration, ethanol *per se* exerts a toxic effect upon the liver, independent of its metabolism.

ACUTE STUDIES

Contradictory results concerning the action of pyrazole administration on acute ethanol-induced fatty liver have been published. Complete prevention of the acute ethanol-induced fatty liver by pyrazole or 4-methyl pyrazole (4-MP) has been reported by some investigators (Morgan and DiLuzio, 1970; Blomstrand and Forsell, 1971), while others found pyrazole to be without effect (Bustos *et al*, 1970). The discrepancy in results has been attributed to:

1. *Time of TG measurement after alcohol administration* (Johnson, Hernell, Fex and Olivecrona, 1971). These authors studied TG accumulation at 6 and 16 hrs after administration of ethanol in combination with pyrazole. They found that at 6 hrs, pyrazole-treated animals showed less TG accumulation than those receiving ethanol alone, but at 16 hrs there was no difference between the two groups. This does confirm in part the study of Bustos *et al* (1970) who measured TG concentrations at 16 hrs. However, it conflicts with the findings of Morgan and DiLuzio (1970) and Blomstrand and Forsell (1971), who reported lower TG levels in the pyrazole-treated animals at 20 hrs after the ethanol.

2. *Sex difference in the animals used* (Domanski, Rifkenberick, Stearns, Scorpio and Narrod, 1971). These authors reported that pyrazole completely prevented the ethanol-induced TG accumulation in male rats (the sex used by Morgan and DiLuzio, 1970), but was very much less effective in females (the sex used by Bustos *et al*, 1970). This,

however, conflicts with DiLuzio's finding of identical results in males and females (personal communication).

3. *Dose of ethanol used* (Nordmann, Ribiere, Rouach and Nordmann, 1972). These authors administered ethanol in doses of 4g/Kg (the dose used by Bustos *et al*, 1970) as well as 6g/Kg (the dose used by Morgan and DiLuzio, 1970). They found that sex of the animals, the dose of pyrazole and the time interval between the administration of ethanol and pyrazole were unimportant, whereas the dose of alcohol was critical. In their studies, pyrazole prevented the ethanol-induced TG accumulation when the dose of ethanol was 6g/Kg, but not when it was 4g/Kg. They therefore explained the discrepancy between DiLuzio and Morgan's finding and ours on the basis of ethanol dose and concluded that hepatic TG accumulation at the lower alcohol dose (4g/Kg) did not depend on ethanol metabolism, while at the higher dose (6g/Kg) it did. This explanation, however, is unsatisfactory since ethanol oxidation is a zero order process at all but very low ethanol concentrations (2-5 mM). Therefore the ethanol metabolism is saturated at doses much below 4g/Kg, so that the increase to 6g/Kg could not affect the rate of ethanol metabolism.

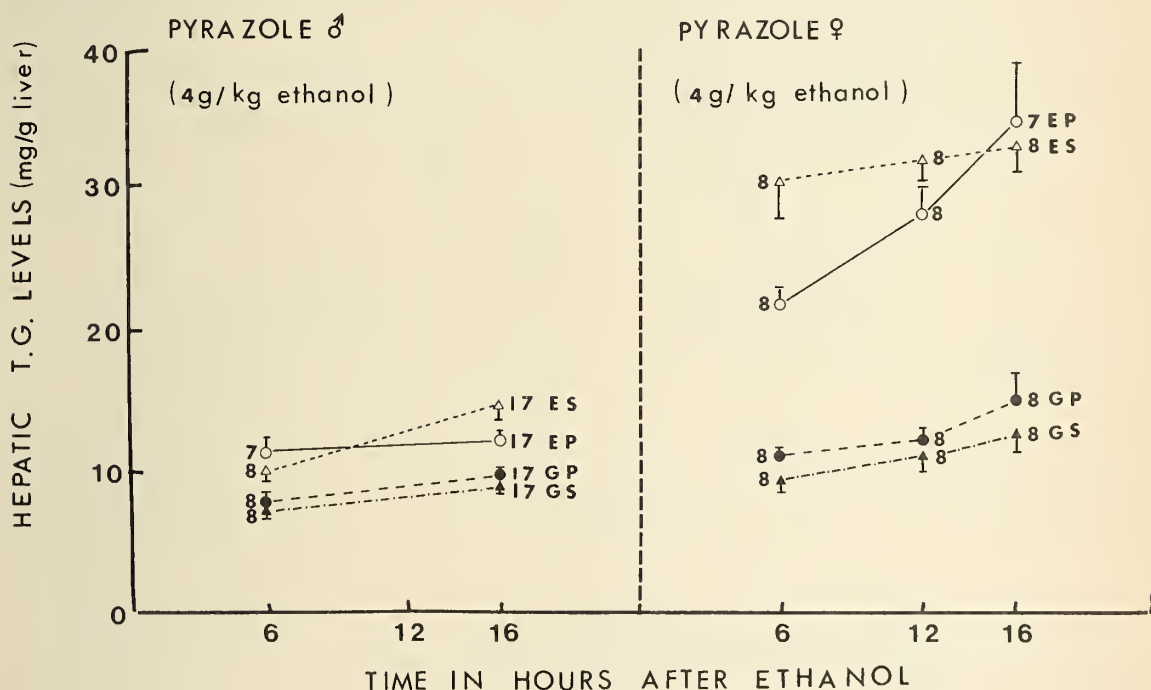


Figure 1. Hepatic TG concentrations in male and female rats at various times after administration of ethanol (4g/Kg; 25% v/v in water, p.o.) in the presence and absence of pyrazole (272 mg/Kg, as a 1.7% solution in saline). In each experiment, the rats were divided into four groups. After a period of 16-17 hours without food, one group (ES) received an I.P. injection of saline, followed 10 minutes later by ethanol. Controls (GS) received the injection of saline, followed by equal volumes of an equicaloric solution of glucose. Two additional groups (EP and GP) received the same doses of ethanol and glucose respectively 10 minutes after the I.P. injection of pyrazole. TG were extracted and measured as described by Bustos *et al* (1970). Vertical lines indicate positive or negative half of standard errors, with the adjacent number indicating the value of *n* for each time.

Studies on acute fatty liver were therefore designed to resolve the above discrepancies¹ and to clarify the relative contribution of metabolism of ethanol and ethanol *per se* to the development of fatty liver.

The hepatic TG concentrations at different times after administration of ethanol (4g/Kg) or isocaloric glucose, in male and female rats pre-treated with pyrazole or saline, are shown in Figure 1. The values for the Glucose-pyrazole (GP) group did not differ from those of the Glucose-saline group (GS) in either sex, at any of the times tested. Thus pyrazole by itself did not increase TG concentration. Ethanol in this dosage, either in the presence of pyrazole (EP group) or in its absence (ES group), produced highly significant increases at all times in hepatic TG when compared with their corresponding GP and GS controls. However, the TG levels in females were significantly reduced in EP compared to ES at 6 hrs ($P < 0.001$), but at 12 and 16 hrs this was no longer significant. This finding agrees with the results of Johnson *et al* (1971). In males, the TG accumulation was small and the peak was not reached until 16 hrs; pyrazole apparently did not influence the ethanol-induced TG accumulation at 6 hrs, but reduced it at 16 hrs ($p < 0.01$).

Figure 2 shows the results obtained with 4-MP. In contrast to pyrazole, 4-MP by itself increased TG content, *i.e.* the TG levels in the Glucose + 4-MP (GMP) groups were significantly higher than in the GS groups. This difference was significant in the females at 6, 12 or 16 hrs ($p < 0.025$, 0.02 and 0.005 respectively) and in the males at 16 hrs ($p < 0.005$). Ethanol again produced its usual effects and the values of TG in the ES and EMP groups were significantly higher than the corresponding GS and GMP controls, at all

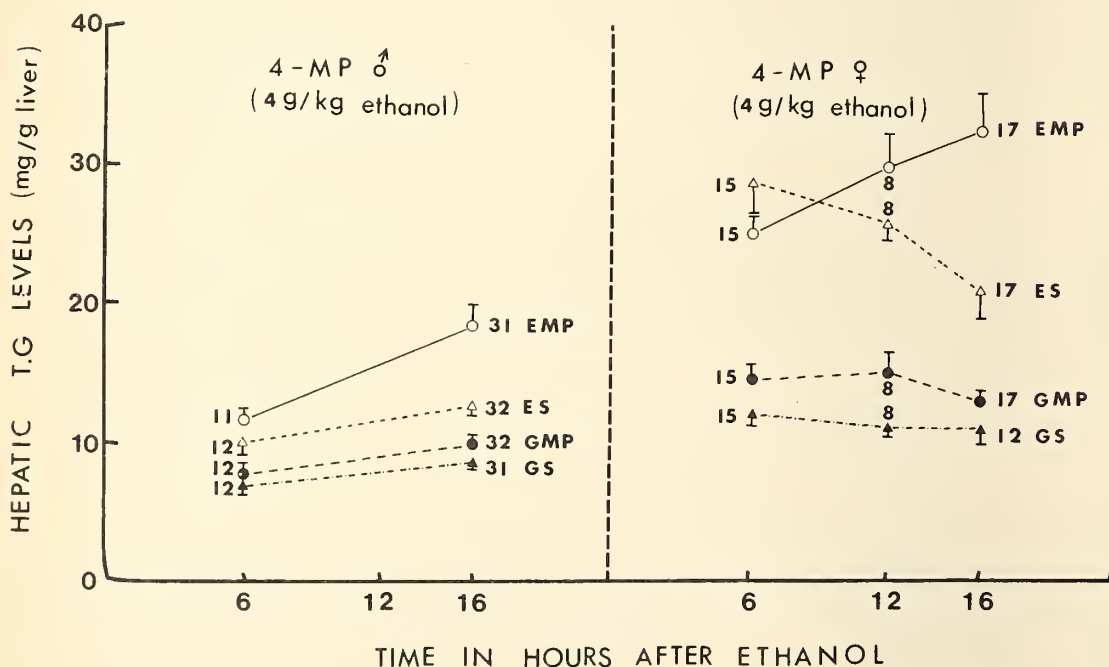


Figure 2. Hepatic TG concentrations at various times in male and female rats after administration of ethanol (4g/Kg) in the presence and absence of 4-MP (200 mg/Kg, as a 1% solution in saline). The experimental protocol and group designations etc. as described in Figure 1, except that the 4-MP groups are designated EMP and GMP.

¹These studies have been described in detail elsewhere (Khanna, Kalant, Loth and Seymour, 1974).

times tested, in both males and females. At 6 hrs, the combination of ethanol with 4-MP gave results similar to those seen with pyrazole, *i.e.* no difference between EMP and ES in males, but a significant reduction in EMP compared to ES in females ($p < 0.005$). The results on TG accumulation at 12 hrs were also similar to those reported above with pyrazole. However, at 16 hrs, in contrast to pyrazole, the TG levels in the EMP group were significantly higher than in the ES group, both in males ($p < 0.005$) and females ($p < 0.001$).

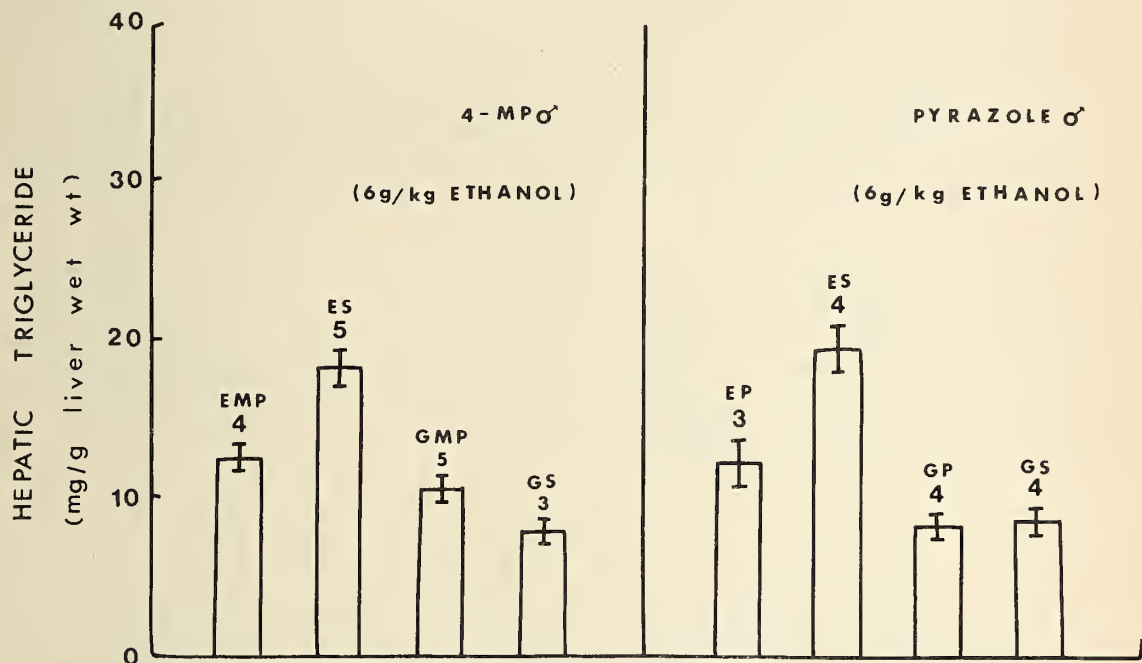


Figure 3. Hepatic TG concentrations at 16 hours in male rats after administration of ethanol (6g/Kg) in the presence and absence of 4-MP and pyrazole. The group designations and experimental protocol as described in Figures 1 and 2. Vertical lines indicate standard errors, with number of animals per group on top of the bars.

Figure 3 shows the results obtained with the larger dose of alcohol (6g/Kg) or isocaloric glucose, in male rats receiving 4-MP or pyrazole. Again, in contrast to pyrazole, 4-MP by itself did increase TG concentration. Furthermore, both 4-MP and pyrazole greatly reduced the alcohol-induced hepatic TG accumulation measured at 16 hrs after ethanol ($p < 0.001$ and 0.02 respectively). This finding is consistent with the observation of Morgan and DiLuzio (1970) and Nordmann *et al* (1971). It emphasizes the difference in pyrazole and 4-MP effects at ethanol doses of 4 vs 6g/Kg.

From the results presented above, it is evident that pyrazole and 4-MP in combination with ethanol produced variable results on hepatic TG accumulation depending on the time of measurement, sex of the animal and the dose of ethanol used. The findings lead us to the following conclusions:

1. Metabolic effects of ethanol do contribute to the development of acute fatty liver, since both pyrazole and 4-MP produced a small but significant decrease in ethanol-induced TG accumulation; this was evident in females at 6 hrs and in males at 16 hrs. The

time difference in the inhibitory effects of pyrazoles may merely reflect a slower process of lipid turnover in the males vs females. Our findings at 16 hrs are in agreement with the results of Domanski *et al* (1971); however, since they did not study TG accumulation at 6 hrs, they were misled into attributing the discrepancy between the results of Morgan and DiLuzio (1970) and ours (Bustos *et al*, 1970) to sex difference.

2. Ethanol, *per se*, independent of its metabolism, also contributes to the production of fatty liver, because the TG values in the EP and EMP groups were markedly higher than their respective GP and GMP groups. If metabolism of ethanol were the sole factor required for the production of fatty liver, then pyrazoles should have completely blocked it, *i.e.* the TG values in the EP and EMP groups should be identical to their respective GP and GMP controls.

3. 4-MP differs from pyrazole in that it independently increases TG concentration. Moreover, its duration of action is considerably shorter than that of pyrazole (Rydberg, Buijten and Neri, 1972; Khanna *et al*, 1974). The combination of these two factors may account, in part, for significantly higher values in the EMP than the ES group at 16 hrs (Fig. 2).

4. The greater reduction in TG accumulation by pyrazole and 4-MP in combination with 6g/Kg dose of ethanol compared to 4g/Kg, seems probably due to marked reduction in blood flow through peripheral adipose tissue and through the viscera, since pyrazole and 4-MP have been shown to have definite CNS depressant effects, which is synergistic with the actions of ethanol (Blum, Geller and Wallace, 1971; Goldberg, Hollstedt, Neri and Rydberg, 1972; LeBlanc and Kalant, 1973).

CHRONIC STUDIES

The studies presented above suggest that ethanol *per se*, as well as metabolic effects of ethanol, contribute to the development of acute alcoholic fatty liver. The question still remains whether under conditions of chronic ethanol ingestion, ethanol *per se* exerts a toxic effect upon the liver, independent of its metabolism. We examined this question by studying the effect of chronic treatment with ethanol, with and without pyrazole, on hepatic TG levels. For this purpose, groups of rats were pair-fed liquid diets containing ethanol or an equicaloric amount of sucrose for a period of 3 - 4 weeks (Lieber, Jones, Mendelson and DeCarli, 1963; Khanna, Kalant and Bustos, 1967). Half of the animals on each diet also received by gavage 37.5 mg/Kg of pyrazole on alternate days. Four separate experiments were carried out. These experiments differed from each other in the diets used. In experiment 1 ethanol provided 35% of the total calories, protein hydrolysate 19%, fat 41% and sucrose 5%. In experiment 2 the ethanol provided only 20% of the total calories, fat 35% and sucrose 25%. The percentage of calories derived from protein was the same as in experiment 1. In experiments 3 and 4, ethanol provided 25 and 35% of the calories respectively. The amount of fat was reduced to 10% and protein increased to 25%, the remainder being balanced with sucrose.

The hepatic TG concentrations after 3 - 4 weeks of administration of ethanol and sucrose diets with and without pyrazole, in four separate experiments, are shown in Figure 4.

Comparison of sucrose-pyrazole (GP) vs sucrose groups (G) revealed that the hepatic TG levels are significantly lower in the GP group than in the G group in experiment 1 ($p < 0.05$) and 2 ($p < 0.001$), but not in experiments 3 and 4. The differential effect of pyrazole in experiments 3 and 4 vs 1 and 2 may be related to a reduction in dietary fat or

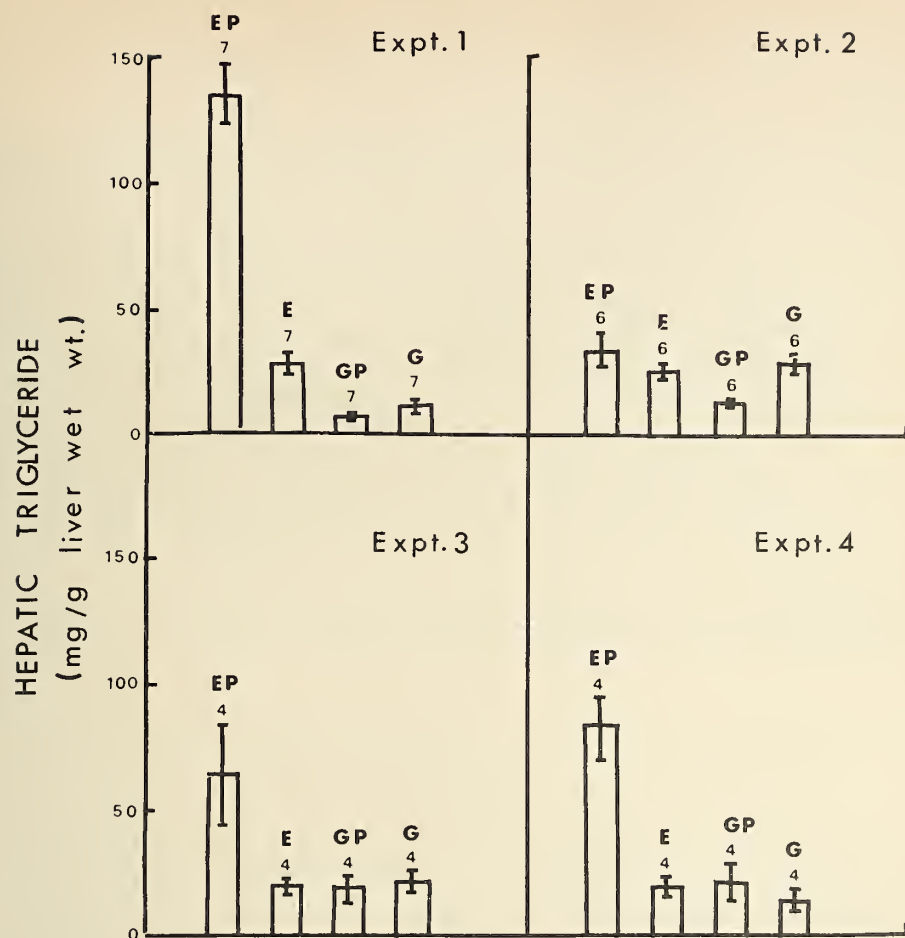


Figure 4. Effect of chronic ethanol treatment, with and without pyrazole, on hepatic TG levels in four separate experiments. Diet and treatments for each experiment are described in the text. The designations EP, E, GP and G refer to the ethanol-pyrazole, ethanol, sucrose-pyrazole and sucrose groups respectively. Vertical lines indicate standard errors, with number of animals per group on top of the bars.

increased dietary protein (Kalant, Khanna and Bustos, 1972). In experiment 1, the ethanol group (E) exhibited a significant increase in TG concentration as compared with the G group ($p < 0.001$). However, in experiments 2, 3 and 4 there was no significant difference in TG levels between the E and G groups. These findings are in essential agreement with previous work in this area (Lieber, Jones and DeCarli, 1965; Porta, Koch, Gomez-Dumm and Hartroft, 1968).

However, the striking finding of these studies was the tremendous increase in TG levels in ethanol-pyrazole (EP) animals in experiments 1, 3 and 4. The TG values in the EP groups were much higher than in the other three groups. However, in experiment 2, the TG values in the EP group were not significantly different from those of the E group. The explanation for this may lie in the low blood alcohol levels achieved in this experiment, even in the presence of pyrazole. The mean level throughout the experiment was 50 mg/100 ml in the EP group, as compared to 15 mg/100 ml in the E group. Obviously, the blood ethanol levels achieved in the E and EP groups in different experiments depend-

ed upon the percentage of ethanol calories in the diet. In experiment 1 they were markedly higher than in the others and in experiments 3 and 4 they were intermediate between those found in experiments 1 and 2.

The total extent of ethanol metabolism must have been the same in animals receiving ethanol alone or ethanol in combination with pyrazole, since there was no progressive alcohol accumulation. Therefore the marked increase in hepatic TG in the EP animals suggests an additional mechanism independent of the metabolism of ethanol. One possible explanation is that longer exposure to higher ethanol concentrations is hepatotoxic. This would be consistent with a direct pharmacological effect of ethanol on the liver cell, as suggested by Leibach (1969). The alternative explanation could be a synergism between some subthreshold hepatotoxic effect of pyrazole and that of ethanol. The latter possibility seems less likely because there was a clear correlation between ethanol concentration achieved and TG accumulation. In experiment 1, the blood alcohol concentration was higher and so was the TG accumulation. In experiment 2, when ethanol provided only 20% of the calories resulting in low blood ethanol levels, pyrazole did not enhance the ethanol-induced TG accumulation. Similar correlation between ethanol concentration and TG accumulation was seen in experiments 3 and 4.

The present results therefore lend at least tentative support to the concept that chronic ethanol exposure has a direct hepatotoxic effect.

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Chronic Effects of Ethanol Pyrazole Administration on Rats Fed a Nutritionally Adequate Diet

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INTRODUCTION

Despite many investigations, there is no fully satisfactory experimental model of human alcoholic liver disease. Acute alcoholic hepatitis, which is considered to be the key lesion in the pathogenesis of human chronic alcoholic liver disease, is also extremely difficult to produce experimentally. Lieber has demonstrated in this Symposium (p. 171) acute alcoholic hepatitis after one year and alcoholic cirrhosis after 4 years of ethanol administration to baboons. This is a highly significant study since it shows that ethanol under nutritionally controlled experimental conditions is cirrhogenic to primates. The baboon model, however, is extremely expensive and time consuming, and such studies are restricted to special primate centers; hence need still exists for other experimental models of alcoholic liver disease.

A major problem encountered in earlier studies of experimental chronic alcoholic liver disease was the introduction of nutritional deficiencies, which alone often sufficed to explain the pathological findings (Best, Hartroft, Lucas and Ridout, 1949; Hartroft, Porta and Gomez Dunn, 1966). Recently, more attention has been paid to methods whereby large amounts of ethanol could be administered without causing nutritional imbalance or deficiencies, and methods whereby prolonged high blood alcohol levels can be obtained in the animals. In studies which utilized the addition of alcohol to the drinking water, the percentage of total caloric intake derived from alcohol was of the order of 20% (see Review by Porta, Koch and Hartroft, 1970). The introduction of a nutritionally adequate liquid diet by Lieber, Jones, Mendelson and DeCarli (1963) allowed for the provision of 36% or more of the total calories as ethanol. This represented a significant advance. However, alcoholics often have much higher blood alcohol levels

than those reached in experimental animals by this technique. Leibach (1969) reported the production of liver cell necrosis and cirrhotic change in rats treated chronically with ethanol in combination with pyrazole, a potent inhibitor of alcohol dehydrogenase. Kalant, Khanna and Bustos (1972) also administered ethanol and pyrazole chronically to rats maintained on a nutritionally adequate diet. They showed that the blood alcohol levels were not only significantly higher than in control pair-fed rats receiving ethanol alone, but remained persistently elevated over a 24 hour period. The hepatic triglyceride levels in these animals were also increased to 3-5 times that found in rats treated with ethanol alone. The present paper describes the effects on hepatic structure of the chronic combined administration of ethanol and pyrazole to rats fed a nutritionally adequate diet.

MATERIALS AND METHODS

Experimental Design

Male rats of Wistar strain, of about 200 g initial body weight, were obtained from High Oak Farms. The animals were caged individually. Each rat was given 80 ml daily of homogenized liquid sucrose diet in graduated drinking tubes as the only source of food and water for one week to permit it to adapt to this form of feeding. After one week, four groups were set up: Group I: Ethanol-pyrazole; Group II: Ethanol; Group III: Sucrose-pyrazole; Group IV: Sucrose. The alcohol-containing liquid diet was modified from that devised by Lieber, Jones, Mendelson & DeCarli (1963) as described by Khanna, Kalant and Bustos (1967). In this diet, ethanol provided 35% of total calories, protein hydrolysate 19%, sucrose 5% and fat 41%. In the control diets, ethanol was replaced isocalorically with sucrose.

Group I received ad lib the ethanol liquid diet in drinking tubes. The amount consumed by each animal was measured every day, and the same amount of the appropriate liquid diets was offered the following day to the corresponding pair-fed animals in the other three groups. The Group I and Group III animals also received by intubation on alternate days 37.5 mg/kg of pyrazole. All the animals were weighed 3 times per week.

The animals were sacrificed in sets of four, *i.e.* the pair-fed animals from each treatment group. They were not starved prior to being sacrificed. Two sets each were sacrificed at 1 week, 2 weeks and 3 weeks for evaluation of the early hepatic lesions. Six sets each were sacrificed at 1 month, 3 at 2 months, 7 at 4-5 months, 4 at 6 months, 1 at 8 months, 2 at 10 months and 1 at 11 months. Thus a total of 120 rats were examined.

Morphological Techniques

Preliminary experiments showed that the ultrastructure of fatty livers was much better preserved by perfusion fixation than by conventional immersion fixation. The following method of perfusion fixation was adopted.

Laparotomy was performed under light ether anaesthesia. A 19-gauge syringe needle was inserted into the portal vein and carefully advanced so that its tip was in the left main branch of the portal vein. The needle had been previously connected via a clinical intravenous infusion set with a flask containing fixative at room temperature. The right portal

vein and the right lobe of liver were tied off with a 00 black suture which was passed between the right and left hepatic lobes. The left lobe of the liver was perfused directly with 1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 or 2 minutes, under a constant hydrostatic pressure of 60 cm of water. Immediately after the perfusion was started, the aorta and inferior vena cava were transected at the level of the renal vein to ensure free flow of fixative. The perfused left lobe instantaneously turned tan and firm, while the unperfused right lobe remained reddish-brown and soft.

Tissue blocks from well perfused areas of the left lobe were transferred directly to 1% osmium tetroxide in phosphate buffer and postfixed for one hour. After dehydration in a graded series of ethanol solutions, the blocks of tissue were embedded in an Epon Araldite mixture. For each rat 10-12 μ sections were cut and stained with toluidine blue. Two blocks each of centrilobular and periportal areas were selected and further trimmed to include 4-5 cell layers from the central vein and 6-7 cell layers from the portal tracts respectively. Ultra thin sections were prepared, stained with lead citrate and examined with a Philips 300 electron microscope.

Slices of unperfused liver from the right lobe were processed for light microscopy, and sections stained with haematoxylin and eosin, Luxol-fast blue, Masson's trichrome, reticulin and oil red O were examined.

Biochemical Techniques

The remaining portions of the right lobe of liver were quickly frozen. Hydroxyproline was measured by the method described by Prockop and Udenfriend (1960) and DNA as described by Munro and Fleck (1966). Both values were expressed as μ g/100 mg of dry defatted liver.

RESULTS

Mortality and Morbidity

As expected from previous studies (Kalant *et al.*, 1972), rats receiving the modified Lieber liquid diet (35% of calories as ethanol) and receiving pyrazole (37.5 mg/kg) on alternate days, maintained their weight and appeared healthy. Animals in the other groups also remained healthy throughout the experiment.

Hydroxyproline and DNA

The analytical results are summarized in Table I. Note that the hydroxyproline levels increased with time between one and six months. However, there was no significant difference in hydroxyproline levels among the four treatment groups at any of the times studied. Both pyrazole and ethanol caused significant reductions in DNA levels at most of the times tested. A summary of the results of an analysis of variance is given in Table II. The results were also expressed as the ratio of hydroxyproline/DNA, as this would be a better indicator of net change in the amount of collagen per cell. The ratio at six months was higher than at earlier times, but there were no significant differences among the four groups at any of the times tested.

TABLE I
HEPATIC HYDROXYPROLINE AND DNA CONCENTRATIONS ($\mu\text{g}/100 \text{ mg}$ DRY DEFATTED LIVER)
IN RATS CONSUMING LIQUID DIETS CONTAINING ETHANOL OR ISOCALORIC SUCROSE
IN PRESENCE AND ABSENCE OF PYRAZOLE*

Time of study (Months)	Parameter measured	Ethanol plus Pyrazole	Ethanol	Sucrose plus Pyrazole	Sucrose
1	Hydroxyproline	$28.73 \pm 3.25(7)$	$24.74 \pm 4.93(6)$	$25.53 \pm 2.60(6)$	$29.95 \pm 3.09(6)$
	DNA	$147.14 \pm 10.32(8)$	$209.57 \pm 16.29(6)$	$175.43 \pm 12.17(5)$	$251.13 \pm 22.02(6)$
	Hydroxyproline/DNA	$0.199 \pm 0.03(7)$	$0.148 \pm 0.04(6)$	$0.135 \pm 0.01(5)$	$0.126 \pm 0.02(6)$
2	Hydroxyproline	$44.35 \pm 6.62(7)$	$41.78 \pm 6.97(4)$	$39.92 \pm 4.52(4)$	$37.84 \pm 5.35(4)$
	DNA	$170.27 \pm 17.48(7)$	$213.23 \pm 36.27(4)$	$212.97 \pm 29.40(4)$	$220.36 \pm 38.29(4)$
	Hydroxyproline/DNA	$0.286 \pm 0.05(7)$	$0.223 \pm 0.06(4)$	$0.203 \pm 0.04(4)$	$0.199 \pm 0.05(4)$
4-5	Hydroxyproline	$41.11 \pm 3.58(11)$	$42.43 \pm 3.89(6)$	$39.49 \pm 4.14(6)$	$43.45 \pm 5.13(7)$
	DNA	$196.81 \pm 9.95(9)$	$242.60 \pm 11.72(7)$	$252.33 \pm 16.64(6)$	$246.99 \pm 13.72(7)$
	Hydroxyproline/DNA	$0.218 \pm 0.03(9)$	$0.174 \pm 0.02(6)$	$0.157 \pm 0.01(6)$	$0.181 \pm 0.03(7)$
6	Hydroxyproline	$67.82 \pm 17.12(4)$	$87.74 \pm 12.42(4)$	$69.24 \pm 6.72(4)$	$73.59 \pm 10.73(4)$
	DNA	$165.92 \pm 17.13(4)$	$210.59 \pm 13.34(4)$	$201.24 \pm 2.61(4)$	$189.44 \pm 10.02(4)$
	Hydroxyproline/DNA	$0.421 \pm 0.11(4)$	$0.418 \pm 0.06(4)$	$0.344 \pm 0.03(4)$	$0.393 \pm 0.07(4)$

*Values shown are mean \pm S.E.M., with number of animals per group in parentheses.

TABLE II
SUMMARY OF THE RESULTS OF ANALYSIS OF VARIANCE

Time of Study and Parameter measured	DF	ETHANOL (EP) + (E) -(SP) - (S)	PYRAZOLE (EP) + (SP) - (E) - (S)	INTERACTION (EP) + (S) -(SP) - (E)	(EP) - (SP)	(E) - (S)	(EP) - (E)	(SP) - (S)
1 MONTH								
HYDROXYPROLINE	15	0.24	1.31	0.82	0.79	0.41	0.35	1.50
DNA	14	2.42*	5.98***	1.06	0.92	2.49*	3.53**	4.75***
HP/DNA	14	1.68	1.47	0.66	1.91	0.88	1.85	0.66
2 MONTHS								
HYDROXYPROLINE	9	0.72	0.18	0.41	0.22	0.80	0.16	0.42
DNA	9	2.06	2.09	1.28	2.36*	0.56	2.38*	0.58
HP/DNA	9	1.56	0.78	0.60	1.53	0.68	0.98	0.13
4 - 5 MONTHS								
HYDROXYPROLINE	16	0.34	1.01	0.23	0.39	0.07	0.85	0.54
DNA	17	2.88*	2.39*	2.38*	3.57**	0.36	3.40**	0.00
HP/DNA	16	1.66	0.35	1.11	1.99	0.39	0.55	1.05
6 MONTHS								
HYDROXYPROLINE	9	0.88	1.69	1.08	0.14	1.39	1.96	0.43
DNA	9	0.53	1.24	2.13	1.88	1.13	2.38*	0.63
HP/DNA	9	1.21	0.55	0.61	1.28	0.43	0.04	0.82

The designations EP, E, SP and S refers to the ethanol-pyrazole, ethanol, sucrose, pyrazole and sucrose groups respectively.

*P<0.05 **P<0.01 ***P<0.001

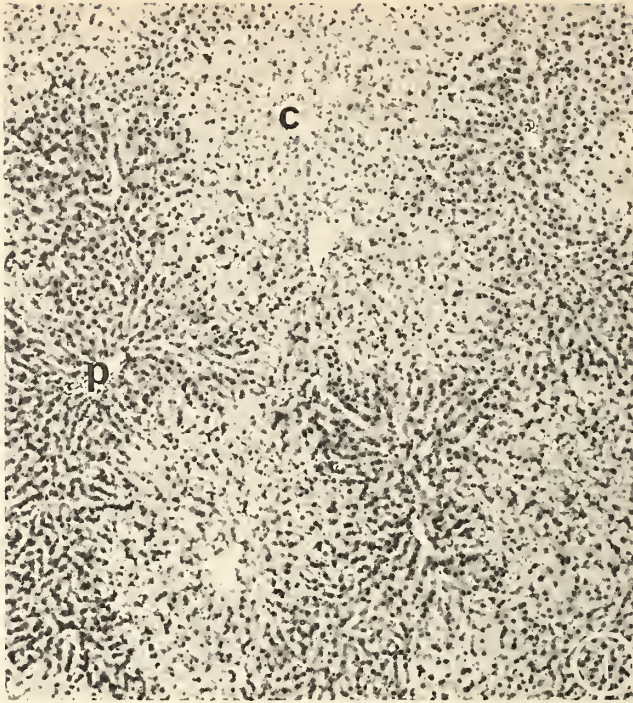


Figure 1. *Ethanol pyrazole 3 weeks. Note marked centrilobular (c) fatty change and normal portal and periportal zones. (p)*

Hematoxylin and eosin X 120

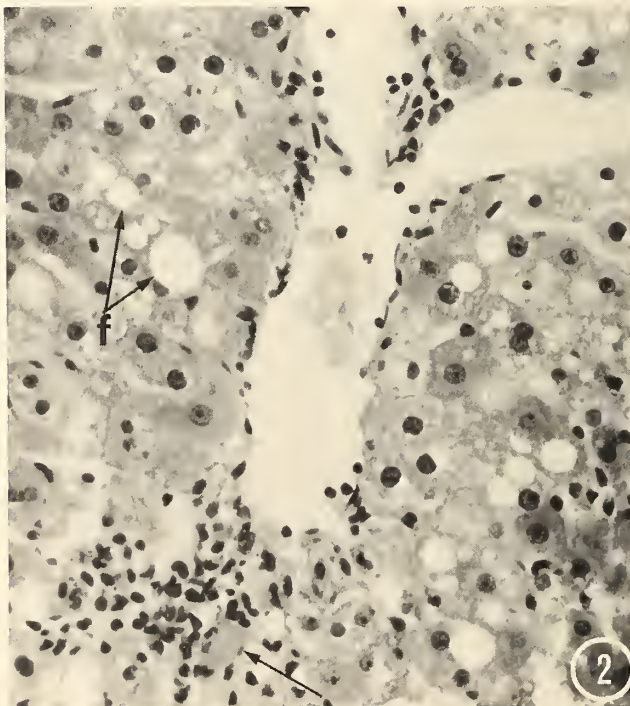


Figure 2. *Ethanol pyrazole group, 4 weeks. Centrilobular fatty change (f), focal necrosis (arrow) of hepatocytes and cellular infiltration predominantly with neutrophils.*

Hematoxylin and eosin X 250

LIGHT MICROSCOPY

Livers from the ethanol-pyrazole treated rats consistently showed marked lipid deposition, the extent of which correlated well with the biochemical finding that the hepatic triglyceride levels were 3-5 times as high as in the ethanol group (Kalant *et al.* 1972). After one week of treatment, lipid droplets were panlobular in distribution. In 2-3 weeks, lipid deposition was strikingly centrilobular and remained so for the remainder of the experimental period (Fig. 1). At 1 month and 2 months, focal degenerative changes were observed in the centrilobular cells. There were individual necrotic liver cells around the central veins and some necrotic foci were infiltrated by polymorphonuclear leukocytes (Figs. 2, 3 and 4). Some of the vacuolated, degenerating centrilobular cells contained irregular, perinuclear eosinophilic hyalin material. Frequently, leukocytes were aggregated in satellite arrangement around extruded hyalin material and necrotic cells in the sinusoids. The hyaline material stained positively with Luxol-fast blue. A phlebitis of the terminal hepatic veins was also present. These centrilobular changes closely resembled those seen in the human hepatic lesion: acute alcoholic hepatitis.

Hepatic cellular necroses and inflammation were *absent* in animals which had received more than 2 months of treatment. There was progressive increase in centrilobular lipid accumulation, but the periportal liver cells remained normal histologically. No fibrosis or cirrhosis was seen.

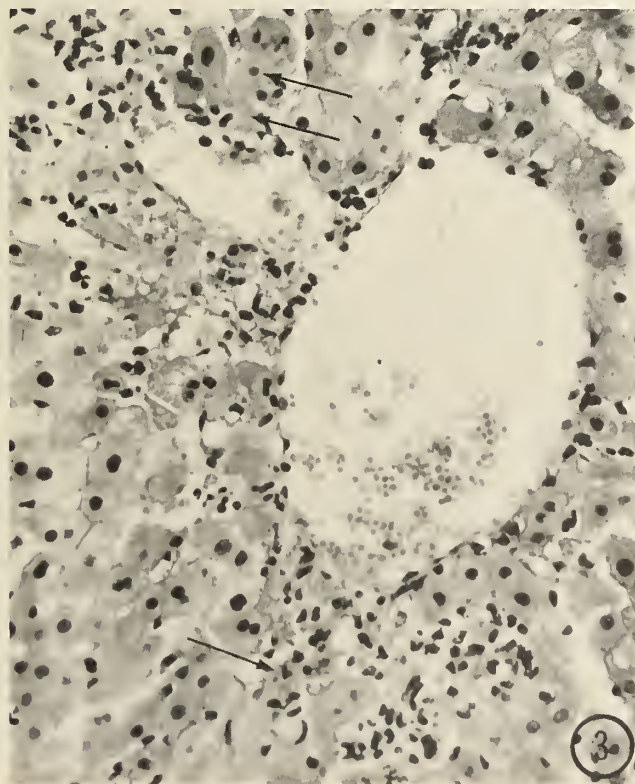


Figure 3. Ethanol pyrazole, 8 weeks. Note centrilobular fatty change, hepatocellular necrosis (arrows) and leukocytic infiltration.

Hematoxylin and eosin X 180

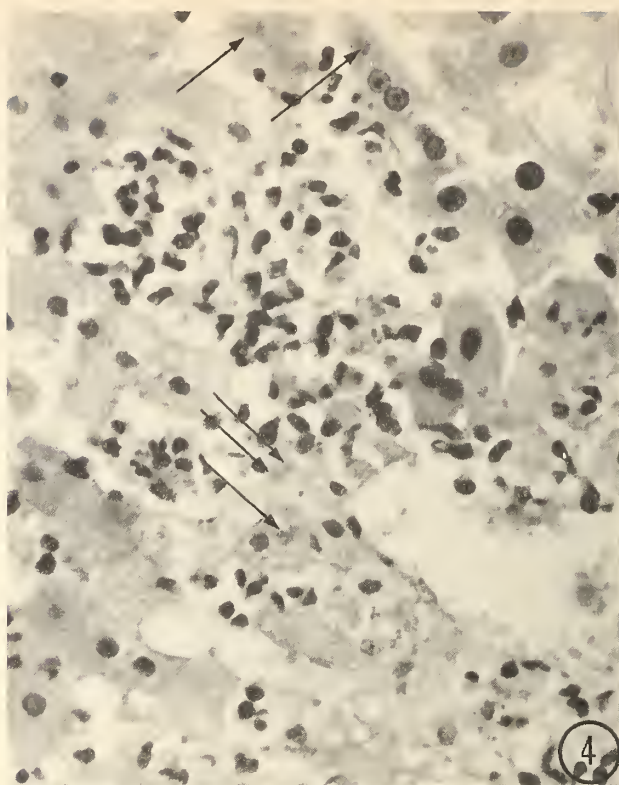


Figure 4. *Ethanol pyrazole, 8 weeks. Higher magnification of part of Fig. 3. Arrows point to cytoplasmic hyalin material and extruded hyalin surrounded by leukocytes.*

Hematoxylin and eosin X 280

Rats treated with ethanol developed fatty liver within 2 weeks of treatment. The extent of lipid accumulation was minimal and generally confined to the parenchymal cells immediately adjacent to the terminal hepatic (central) vein. The periportal and midzonal cells were normal, and there was no cellular necrosis, leukocytic infiltration or fibrosis.

The hepatic histology in pyrazole-sucrose and sucrose treated animals was normal throughout the entire period of the experiment.

ELECTRON MICROSCOPY

Ethanol-Pyrazole Treated Rats (Group I).

Hepatocellular changes were always more marked in centrilobular cells. In virtually all these hepatocytes, lipid was present in variable amount. The fat droplets were non membrane-bound and had the ultrastructural appearance of lipid composed predominantly or entirely of triglyceride. Mitochondria showed mild pleomorphism; no megamitochondria were seen but some showed increase in matrical material.

The rough endoplasmic reticulum (RER) was always abnormal. The typical stacks of RER cisternae of normal hepatocytes were totally absent; instead, the entire rough endoplasmic reticulum was vesiculated (Figs. 5, 6 and 7). In many of the coated vesicles, slightly electron dense material of either protein or lipoprotein type was evident. Free ribosomes appeared greatly reduced in number; they also lacked their normal helical or polysome arrangement, and occurred singly or in pairs.

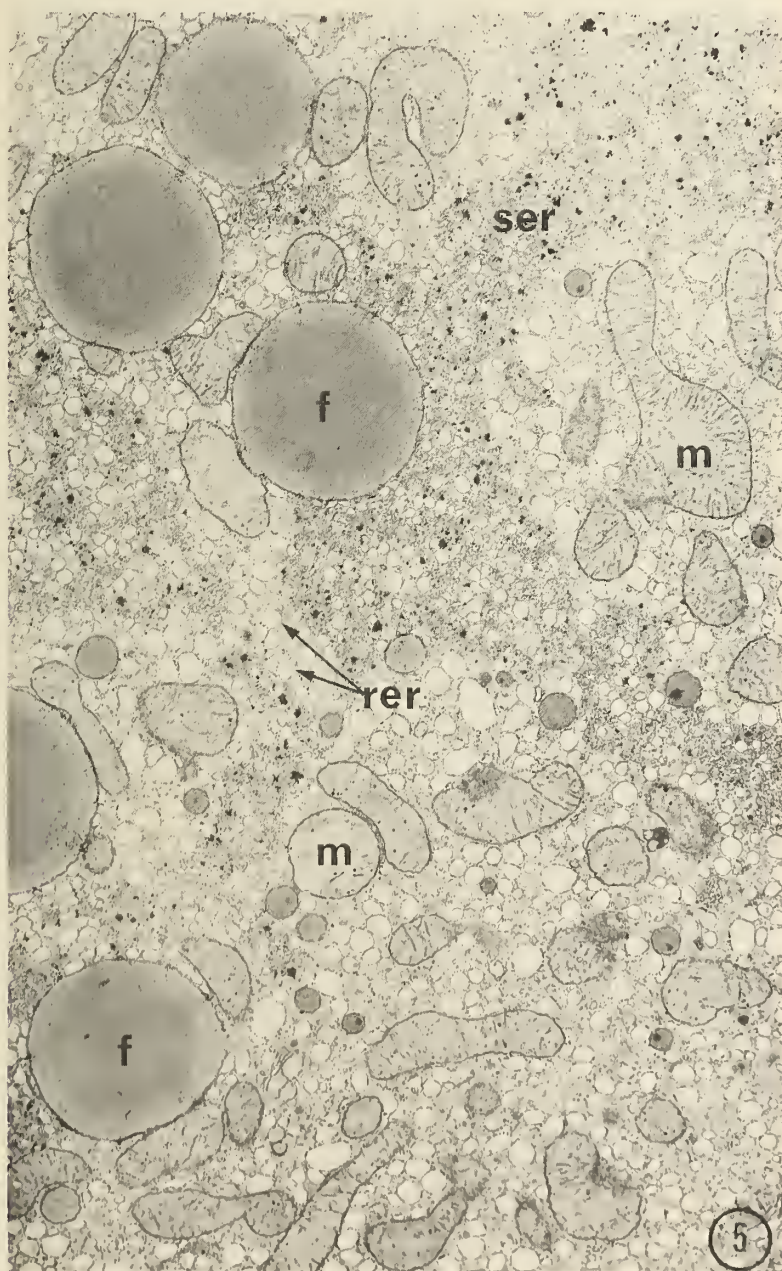


Figure 5. *Ethanol pyrazole, 4 weeks. Fat droplets (f) and proliferated SER (ser) are conspicuous. Glycogen is sparse. Mitochondria (m) are of variable shape, some are enlarged. The rough endoplasmic reticulum (rer) is vesiculated.*

Lead citrate X 25000



Figure 6. *Ethanol pyrazole, 4 weeks.* Note vesiculated RER, (rer), absence of cisternae, reduction of free ribosomes and dispersal of ribosomal aggregates. The Golgi vesicles (G) and some ribosome coated vesicles contain lipoprotein droplets. The SER (ser) is hypertrophied.

Lead citrate X 25000

The smooth endoplasmic reticulum (SER) was notably hypertrophied in these cells. Confluent nodular masses of their proliferated smooth membranes occupied large portions of the cytoplasm. Cytolysosomes and focal cytoplasmic degradation were observed. Glycogen particles were sparse or absent. Golgi, peroxisomes, cell membranes, bile canaliculi and other structures appeared unaltered. Many centrilobular hepatocytes showed

advanced degenerative changes particularly involving the endoplasmic reticulum. In such cells, the SER formed tightly clumped masses of smooth vesicles and the rough endoplasmic reticulum showed marked vesiculation (Figs. 5 and 6).

In cells which by light microscopy contained irregular clumps of eosinophilic granular material, large irregular cytoplasmic aggregates of membranous material were noted by

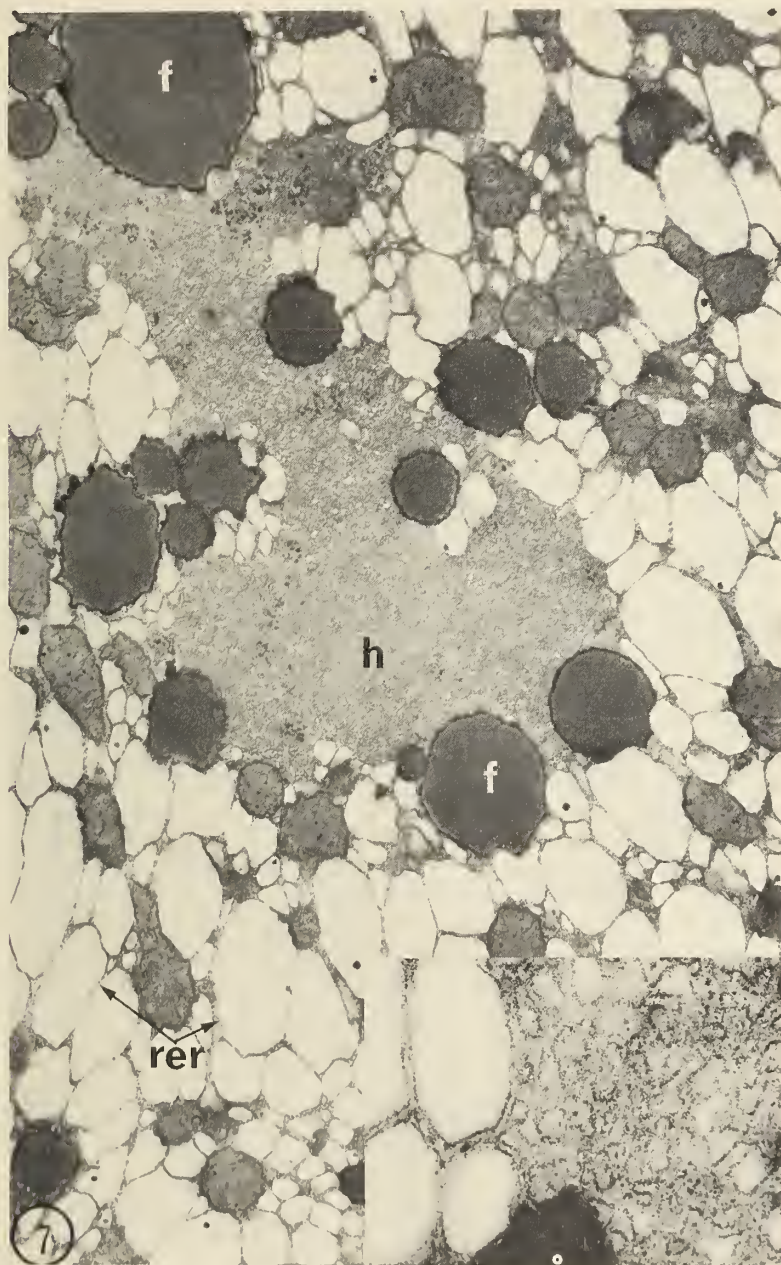


Figure 7. *Ethanol pyrazole, 8 weeks.* This hepatocyte shows marked RER vesiculation (*rer*), and fat accumulation (*f*). The central part of the micrograph is occupied by a conglomerate mass of proliferated SER membranes corresponding to the hyalin (*h*) material seen by light microscopy. The insert shows the membranous nature of this lesion at higher magnification.

Lead citrate X 20500

Insert X 51300

electron microscopy. These masses had the overall configuration of so-called alcoholic hyalin; they appeared to be derived from degeneration of the proliferated smooth endoplasmic reticulum (Fig. 7). Some cells containing these hyalin masses were necrotic as judged by their total loss of ribosomes and extreme distention of their rough endoplasmic reticulum (Fig. 7). Cells of similar appearance were noted in the areas of leukocytic infiltration; phagocytosed necrotic cellular debris was noted in macrophages in these

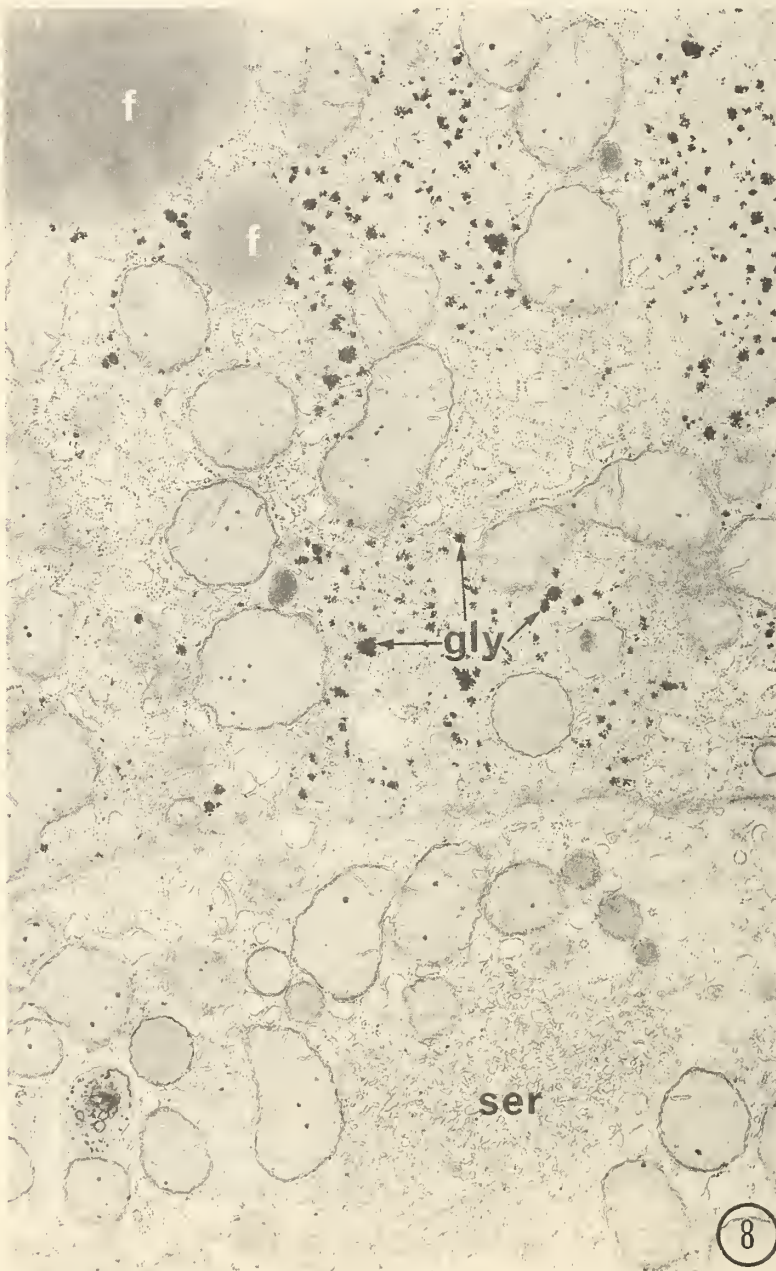


Figure 8. *Ethanol, 8 weeks. Centrilobular hepatocyte showing fat droplets (f), and mild SER (ser) proliferation. Mitochondria are mildly enlarged. Short RER cisternae are present as well as free ribosomal aggregates. The black particles are glycogen rosettes (gly).*

areas. In two animals, collagen deposition was noted in the wall of the terminal hepatic veins and sinusoids.

In contrast to these marked changes in centrilobular hepatocytes, periportal liver cells were normal or near normal in fine structure. Lipid droplets were small. RER cisternae were disposed in regular parallel stacks, although vesicular forms were also encountered in some cells. Free ribosomes and polysomes were abundant in the cytoplasm. SER showed minimal proliferation, glycogen was abundant and other cellular organelles entirely normal.

Ethanol Treated Rats (Group II)

Centrilobular hepatocytes contained lipid droplets. The RER cisternae were decreased, shortened and dilated, especially at their extremities. Vesiculation of the RER was present, but in some parts of the cells the lamellar arrangement of the RER cisternae was preserved. Dilated RER cisternae were seen encircling mitochondria. There was no ribosomal detachment from the RER cisternae or vesicles and polysomal aggregates were abundant in the cytoplasm. The SER was proliferated and consisted of scattered networks of loosely arranged, narrow anastomosing tubules often associated with glycogen deposits (Fig. 8). Most of the mitochondria were normal in appearance; a small proportion of them were swollen, had rarefied matrices and blunting of cristae. Breaks in the outer mitochondrial membrane were not observed. Electron-dense spherical particles were present in the terminally dilated saccules and associated vesicles of the Golgi appearance. Lysosomes were increased and focal cytoplasmic degradation was present. Peroxisomes were normal.

Compared with the controls, the SER in the periportal hepatocytes was increased. In other respects, however, the ultrastructure of the periportal cells was normal and lipid deposits were absent.

Sucrose-Treated Rats (Groups III and IV)

The ultrastructure of the hepatocytes was normal throughout the period of the experiment. Compared with the periportal cells, the centrilobular hepatocytes had more abundant smooth endoplasmic reticulum (SER), fewer elongated stacks of RER and fewer free ribosomes in the cytoplasm, as is normally seen.

No ultrastructural abnormalities were observed in the hepatocytes of pyrazole-sucrose treated rats (Fig. 9).

DISCUSSION

Pyrazole-Alcoholic Hepatitis

Alcoholic liver disease in man comprises a spectrum of pathological lesions which include fatty liver, acute alcoholic hepatitis, central hyaline necrosis, central hyaline sclerosis, septal and diffuse hepatic fibrosis and cirrhosis of micronodular, macronodular, or mixed

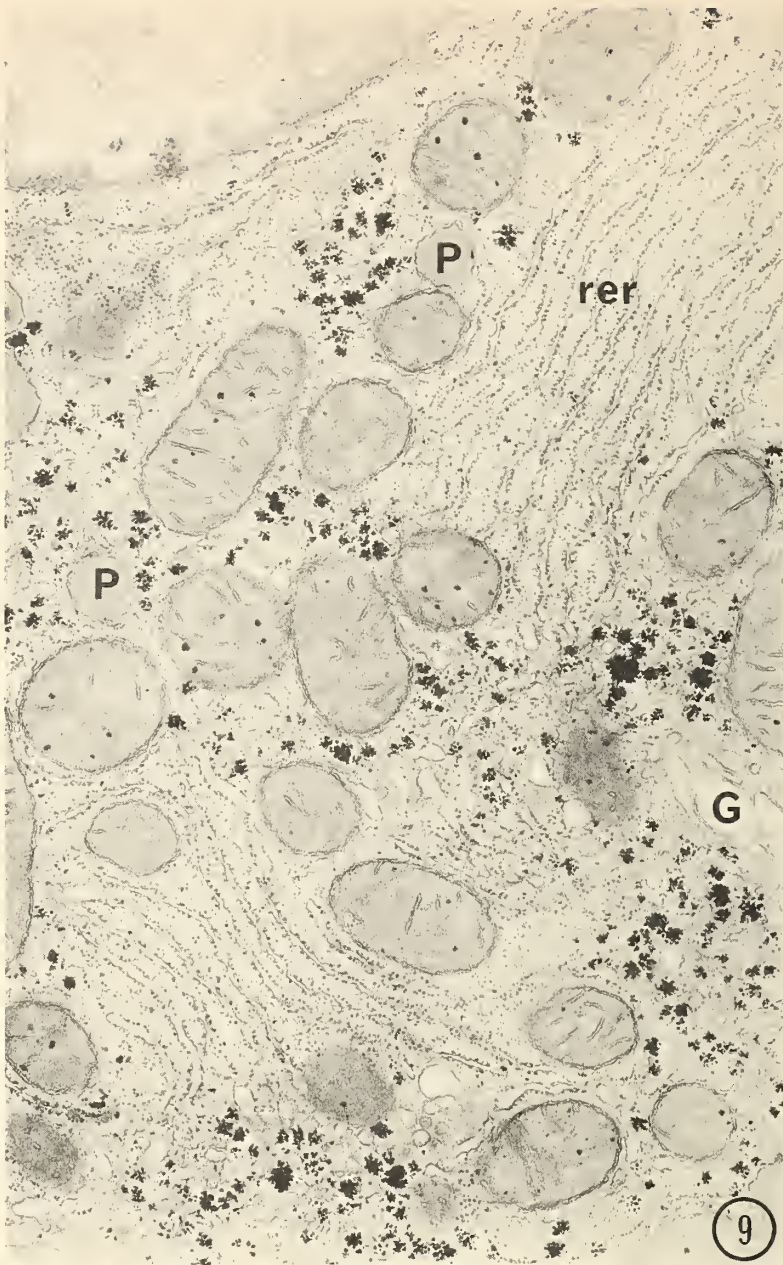


Figure 9. *Pyrazole, 8 weeks.* Note stacks of RER cisternae (rer). Golgi (G), SER, mitochondria, peroxisomes (p) are also all normal.

Lead citrate X 25000

types (Rubin, 1973). Fatty liver has been very extensively investigated but is no longer considered the precursor of cirrhosis in the chronic alcoholic (Gerber and Popper, 1972). Recent investigations indicate that acute alcoholic hepatitis (Beckett, Livingston and Hill, 1961; Porta, Bergman and Stein, 1965; Edmondson, Peters, Frankel and Borowsky, 1967) is of key importance in the pathogenesis of chronic alcoholic liver disease in man. From human liver biopsy studies, it has been shown that this lesion is the precursor of central hyaline sclerosis, portal hypertension, septal and diffuse fibrosis and cirrhosis in

the chronic alcoholic (Gerber and Popper, 1972; Reynolds, Hidemura, Michel and Peters, 1969; Edmonston, Peters, Reynolds and Kuzma, 1963).

The lesion is inflammatory and may or may not be associated with fat or cirrhosis (Rubin, 1973); essential features are liver cell degeneration and necrosis, so-called alcoholic hyalin, leukocytic infiltration and a centrilobular location. Frequently these changes are accompanied by fibrosis which obliterates the central (terminal hepatic or efferent hepatic) vein branches and neighbouring sinusoids. Acute alcoholic hepatitis has never been reported in small laboratory animals, aside from the possible exception of the lesion illustrated by Takeuchi, Takada, Kato, Hasumura, Ikegami and Matsuda (1971) in rats which were fed alcohol chronically and, in addition, given intermittent large supplementary doses by stomach tube.

From a structural viewpoint, therefore, one of the most interesting results of the present study was the centrilobular hepatitis that was seen in ethanol-pyrazole treated rats. The massive hepatocellular necrosis and fibrosis induced with ethanol and pyrazole by Lelbach (1969) does not resemble the lesion seen in the present study, in which dietary factors were carefully controlled and in which the dose of pyrazole was half that used by Lelbach (37.5 mg/kg/2 days, compared with 25-50 mg/kg/day). The hepatitis seen in this study is similar by light microscopy to human alcoholic hepatitis. It is centrilobular, the efferent (terminal hepatic or central) veins are involved, there is focal hepatocellular necrosis, leukocytic infiltration and satellitosis — all typical features of the human lesion — and it is accompanied by fatty change.

In addition, some hepatocytes contained eosinophilic clumped perinuclear material that by light microscopy bore resemblance to alcoholic hyalin. This material has the same tinctorial characteristics as alcoholic hyalin and is positively stained by Luxol-fast blue. By electron microscopy, typical microfilamentous alcoholic hyalin, as observed in human biopsies, was not found. The eosinophilic hyaline material appeared to correspond to proliferated, clumped and often necrotic SER. The origin and nature of human alcoholic hyalin (Biava, 1964; Flax and Tisdale, 1964; Schaffner and Popper, 1965; Smuckler, 1968) is still disputed; it is not specific to alcoholism nor is it specific to liver. Moreover, several morphological types of alcoholic hyalin have recently been described (Yokoo, Munick, Batti and Kent, 1972). There is now general agreement that it is unrelated to megamitochondria (Iseri and Gottlieb, 1971). Yokoo *et al.* (1972) suggested that it could be part of a contractile protein system elaborated as a result of alcoholic hepatic injury. The most recent view is that of French and Davies in this Symposium (see p. 113), namely, that it is fibrillar but not contractile. The hyaline material in "pyrazole-alcoholic hepatitis", as the present experimental lesion might best be termed, is membranous rather than fibrillar. Whether this is due to species differences or not remains unknown.

Also in the rat, the "pyrazole-alcoholic hepatitis" does not progress to cirrhosis. On the contrary, the lesion appeared at one and two months of combined ethanol-pyrazole administration but was not seen in animals which had received the same treatment for more prolonged periods. It is also noteworthy that in these animals, the hepatic fatty change progressed up to the first month of the experiment and then stabilized. These findings suggest that adaptive changes had taken place in these animals.

Zonal Distribution of Ethanol Lesion

The ultrastructural changes in the liver of animals following chronic alcohol consumption have been well documented (Rubin, Hutterer and Lieber, 1968; Dobbins, Rollins, Brooks

and Gallon, 1972; Gordon and Lough, 1972; Ruebner, Brayton, Freeland, Kanagama and Tsao, 1972). Fatty change, mitochondrial swelling, dilatation of rough endoplasmic reticulum cisternae, reduction in the RER and proliferation of the SER are encountered. Similar findings are recorded in human alcoholics (Sternlieb, 1967; Porta, Bergman and Stein, 1965; Schaffner, Loebel, Weiner and Barka, 1963) and in human volunteers given ethanol (Rubin and Lieber, 1967). Dobbins *et al.* (1972) reported that in rats chronically consuming alcohol, the hepatocytic SER was decreased; Rubin, Hutterer and Lieber (1968) on the other hand, found proliferation of the SER. In the present experiment, proliferation of the SER was observed in the ethanol and ethanol-pyrazole treated rats.

It is of importance in studies of this type to assess the zonal distribution of the changes. In virtually all the reports cited, the effects of chronic alcohol administration are more severe in centrilobular hepatocytes; in the study by Dobbins *et al.* the centrilobular cells were excluded from analysis. This may be an important factor in explaining the discrepancy between their results and those of other investigators. In the present experiment, the ethanol-pyrazole treated animals showed more severe changes than those treated with ethanol alone. Marked fatty change appeared early and persisted in centrilobular and midzonal hepatocytes, whereas periportal hepatocytes showed minimal fatty change only.

The reason for the centrilobular localization of the hepatic lesion in alcoholic liver injury is not known. The difference in structural and enzymatic endowment of these cells may be important. Greenberger, Cohen and Isselbacher (1965) in a histochemical study reported that alcohol dehydrogenase was present predominantly in the periportal hepatocytes. However, this claim has been disputed, so that a firm assertion cannot be made (Morrison and Brock, 1967). Therefore the possible role of alcohol metabolism, and of its inhibition by pyrazole, in determining the localization of liver injury, is not yet clear.

Hepatotoxicity of Alcohol

The histological and ultrastructural changes in the ethanol-pyrazole treated rats are similar to, but significantly more severe than, those seen in animals receiving ethanol alone. This observation, when correlated with the known persistently high blood alcohol levels in the ethanol-pyrazole treated group (Kalant *et al.*, 1972), supports the view that prolonged exposure to unmetabolized alcohol is hepatotoxic, quite apart from the effects of alcohol metabolism in the liver. However, the difficulty to establish this conclusively in a chronic experiment is recognized.

To study the effects of ethanol per se, as opposed to those of ethanol metabolism, the metabolic inhibitor used should ideally produce a complete block in ethanol breakdown and should itself be non-toxic. However, complete inhibition of alcohol metabolism would lead to progressive and rapidly fatal blood alcohol levels. Moreover, it is known that pyrazole does have effects other than those on alcohol dehydrogenase. Pyrazole has been shown to alter mitochondrial structure and function (Rubin, Beattie, Toth and Lieber, 1972; Lieber, Cederbaum and Rubin, 1973) and to inhibit the microsomal ethanol-oxidizing system (MEOS) and microsomal drug metabolizing enzymes (Lieber, Rubin, DeCarli, Misra and Gang, 1970; Rubin, Gang and Lieber, 1971). Structural changes have also been noted including vesiculation of the smooth endoplasmic reticulum, scarcity of the rough endoplasmic reticulum, mitochondrial enlargement and distortion (Lieber *et al.*, 1970). However, in the present study the pyrazole-treated animals received a dose (37.5 mg/Kg/2 days) only one quarter of that used by Lieber and co-workers

(1970) (65 mg/Kg/day). It should also be noted that by both light and electron microscopy, no structural abnormalities were present in pyrazole-treated animals in the present study. Nonetheless, theoretically the pathological changes could be the result of alcohol potentiating some latent toxic effects of pyrazole, although this is considered unlikely. Further studies into the possibility that alcohol per se is hepatotoxic, using other experimental approaches, are warranted.

SUMMARY AND CONCLUSIONS

Chronic ethanol-pyrazole administration to rats results in marked fatty change predominantly in the centrilobular regions of the liver. After one or two months, a centrilobular hepatitis appears that closely resembles human alcoholic hepatitis; it is termed "pyrazole-alcoholic hepatitis". Fibrosis and cirrhosis were not produced. By electron microscopy, the most significant structural abnormality seen involved the endoplasmic reticulum; the agranular reticulum was hypertrophied but the rough component showed marked disarray, diminution, dispersion and degeneration. Pyrazole alone produced no histological or ultrastructural changes in the dosage used in this study. The results of this study suggest that alcohol per se is hepatotoxic and is responsible for the marked structural changes seen in ethanol-pyrazole treated animals.

ACKNOWLEDGEMENT

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Some Fundamental Aspects of Liver Injury

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INTRODUCTION

The response of patients in chronic alcoholism encompasses many of the common pathologic reactions seen in the liver in disease. Included among these are fatty liver, necrosis, nodular regeneration and fibrosis. In my view, the understanding of "alcoholic liver pathology" is clearly dependent upon the development of new insight into the fundamental mechanisms responsible for these various pathologic reactions in the liver.

In this paper I shall discuss what we know about pathogenesis of some of these response patterns of the liver as a background to an indepth exposition of the state of the art today in alcoholic liver injury. I plan to concern myself with the biochemical basis for the development of fatty liver and liver cell necrosis, with some aspects of regeneration and enzyme induction as important components in liver disease, and to present some questions concerning cirrhosis and its pathogenesis. Throughout this presentation, the major emphasis will be on mechanisms, not phenomena, since, in my view, this is where we are so far behind. I am convinced that only through clarification of chemical and molecular as well as cellular mechanisms can many of the contradictions, paradoxes and difficulties associated with the analysis of "alcoholic liver pathology" be resolved and possibly even solved. Although I naturally will draw heavily upon the published work of many investigators including my former and present colleagues, I intend also to present some less orthodox notions about some aspects of liver disease. Any perusal of the literature, no matter how cursory or how exhaustive, leads one to the conclusion that we are in desperate need of new thoughts and new approaches to this age old problem of man. Any contributions, however small, are welcome.

FATTY LIVER

Our knowledge concerning fatty liver is much more advanced than that of any other pathologic process in the liver. This is no doubt due to its relative simplicity in comparison to other lesions and to our considerable knowledge about the liver and lipid metabolism (e.g. Farber, 1973a).

The liver plays an important role in the synthesis of fatty acids (FA) from carbohydrate and amino acids, in the production of ketone bodies during the oxidation of fatty acid and in the preparation of lipids, especially triglyceride (TG), for transport to all organs and tissues of the body including the adipose tissue (Fig. 1).

Almost all fatty livers are due to the accumulation of TG. TG, suitably packaged with protein, phospholipid, cholesterol, cholesterol esters and carbohydrate, is the major form in which fatty acids from any source are transported from the liver to the periphery. The fatty acids may come from dietary lipids, from carbohydrate and amino acids and from the adipose tissue. Naturally, the level of TG at any time is the result of the balance between the rates of exogenous supply and of endogenous synthesis of fatty acids on the one hand and the rate of TG secretion as very low density lipoprotein (VLDL) and perhaps as other lipoproteins on the other.

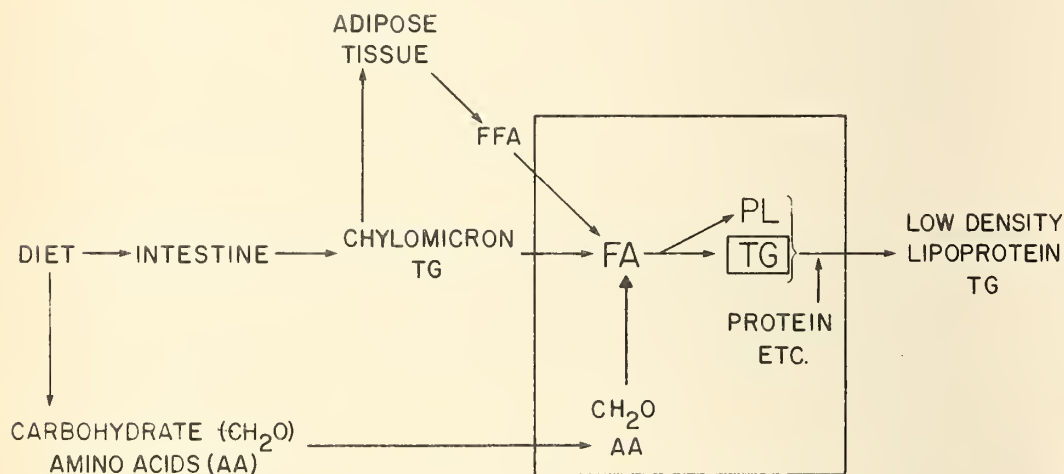


Figure 1. Diagrammatic representation of major sources of fatty acids for TG synthesis in the liver. In the alcoholic, an additional possible source of fatty acids is ethanol itself or one or more of its derivatives.

Mechanisms

One pathogenetic mechanism of fatty liver is the "oversupply" of fatty acids from outside the liver. If the rate of supply is greater than the maximum rate of secretion of TG, the TG will accumulate and a fatty liver will ensue. This appears to be a major mechanism in the fatty livers induced by excess levels of some drugs or hormones which cause lipolysis in adipose tissue.

Conceivably, this mechanism of relative oversupply of FA could play an important role in the genesis of the acute fatty liver in the experimental animal given one or a few doses of ethanol during a short period of fasting, as studied by DiLuzio (1958); Lieber and Schmid (1961); Horning *et al.* (1960); Nikkila and Ojala (1963) and Stein and Stein (1965) and more recently by others (see Stein, Bar-On and Stein, 1972; Lieber, 1968 and Isselbacher and Greenberger, 1964). Under these conditions, TG, first as small liposomes (Baglio and Farber, 1965; Stein and Stein, 1965; Novikoff, Roheim and Quintana, 1966) and later as larger collections of lipid, accumulate quite rapidly in the liver cell and also in the space of Disse. The findings in the acute phase of the experimental model are consistent with the hypothesis of oversupply of FA in the presence of a maximum rate of TG-synthesis (Fig. 2). The pathway for TG secretion to the exterior seems to be fully saturated with apparent lipoprotein particles in the Golgi apparatus and outside in the space of Disse. It would appear that the rate-limiting step under these experimental conditions is the "packaging" of TG for export with consequent accumulation of TG at the site of synthesis, the "ends" of the endoplasmic reticulum (ER). Since under these conditions of fasting, the only major site of origin for FA is the blood free FA (FFA) coming from the adipose tissue, TG accumulating in this acute model of ethanol fatty liver is derived from FA outside the liver. Thus, the acute fasted animal given ethanol seems to have an imbalance between the rate of synthesis and the rate of further metabolism of TG and this could account for the rapid induction of a fatty liver (Fig. 2). The normal or elevated levels of plasma TG and lipoproteins are consistent with this hypothesis.

It should be emphasized that this mode is very much dependent upon the physiologic state of the animal. Once food is introduced into this model, the entire pathogenic pattern would change and become more complex. The supply of dietary FA and the *de*

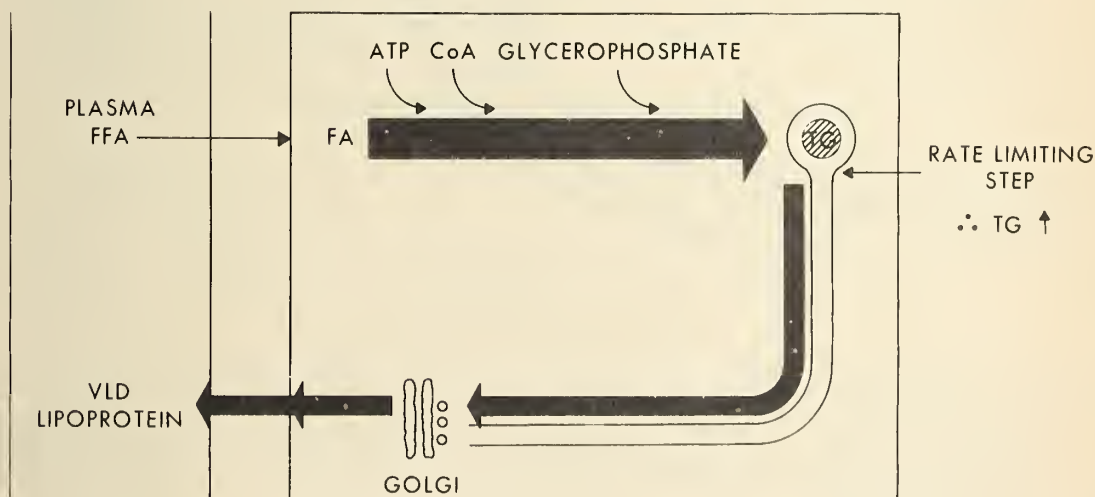


Figure 2. Diagrammatic representation of the major pathway in the liver for TG synthesis and TG secretion as very low density lipoprotein after the acute administration of ethanol in the fasting animal. The width of the arrows is meant to represent the rate of that overall reaction. The major factor in the pathogenesis of this fatty liver is the imbalance between the rate of TG synthesis and the rate of utilization of TG for VLDL synthesis and secretion.

*nov*o synthesis of FA from carbohydrate and amino acid precursors could radically alter the "lipodynamics" and as a result, alter the manifestations of whatever basic lesion ethanol is inducing.

A second pathogenic mechanism of fatty liver is the interference in various ways with the synthesis or intracellular transport of lipoprotein(s) responsible for carrying the major amount of TG in the presence of a "normal" or accelerated rate of TG synthesis. This is the best understood group and includes the fatty liver induced by choline deficiency, ethionine, CCl_4 , puromycin, phosphorus, orotic acid and cerium (Fig. 3) (see Stein, Bar-On and Stein, 1972; Lombardi, 1966, 1971; Farber, 1966, 1967, 1971) and by methionine in the guinea pig (Hardwick *et al.*, 1970; Shinozuka *et al.*, 1971(a), 1971(b)). Interferences with protein synthesis, lecithin synthesis and possibly the synthesis of cholesterol, cholesterol esters or the carbohydrate moiety of very low density lipoprotein may each be reflected in TG accumulation because of their common effects on the synthesis and intracellular transport of lipoprotein in the liver or the secretion into the blood. Although a similar mechanism has occasionally been discussed for ethanol-induced fatty liver, the evidence for this is not convincing.

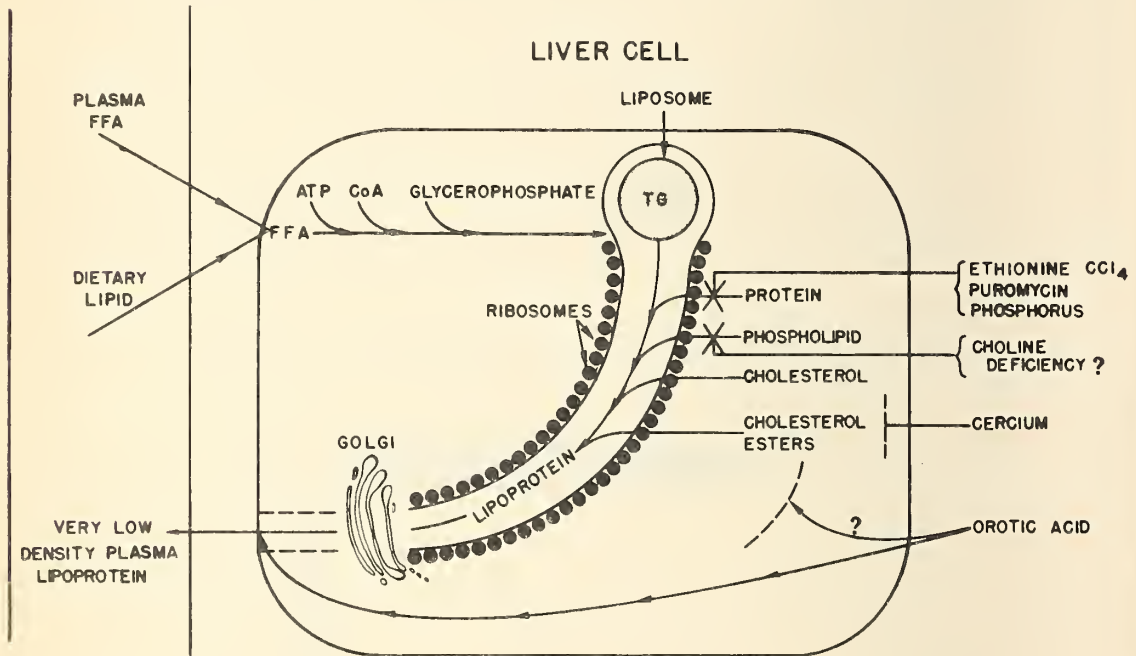


Figure 3. Diagrammatic representation of the pathogenetic mechanism for one of the major forms of fatty liver. The probable site for interference with the packaging, transport or secretion of TG as very low density lipoprotein by the various hepatotoxins is indicated.

A third pathogenic mechanism for fatty liver is an oversupply of FA from non-lipid precursors inside the liver — carbohydrate, protein and possibly carbon from ethanol itself or one of its metabolites. This naturally has some similarities with the first mechanism in that the major factor in the accumulation of TG is again an imbalance between the rate of synthesis of TG and its rate of utilization for lipoprotein synthesis. However, unlike the other mechanisms, we introduce into this model a whole network of enzyme systems including their controls that generate fatty acids. Biotin and pantothenic acid as

well as other vitamins, etc. play a critical role in this phase of metabolism. In liver, the major site for FA synthesis is the soluble compartment of the cell, the cytosol. Two basic enzymes are involved in the synthesis from acetate or acetyl-CoA — acetyl-CoA carboxylase, which generates malonyl-CoA, and fatty acid synthetase, a multienzyme complex which catalyzes, in a cyclic fashion, the condensation of 7 molecules of malonyl-CoA with acetyl-CoA to produce 1 molecule of palmityl-CoA (see Volpe and Vagelos, 1973). Both enzymes are subject to many controls including, as expected, the nutritional state of the host. For example, fasting or feeding a high fat diet causes a large decrease in enzyme activity while the reverse occurs on feeding a fat-free diet.

Since ethanol has been shown to increase the rate of synthesis of fatty acids in the liver (e.g. see Lieber, 1968; Krebs, 1968), and since the activities of the basic machinery for this effect is so subject to physiologic control, especially dietary, one would expect that the pathologic consequences of ethanol on the liver may be very much dependent upon the physiologic state of the host. It seems to me that very carefully controlled nutritional and other physiologic conditions are absolutely essential if we are to understand the mechanism or mechanisms of genesis of ethanol induced fatty liver in both animals and humans. Although the design of some studies has taken this important factor into consideration, this cannot be said about many studies both in man and in animals. The variations in physiologic or pathologic state of the host could well account for the confusion that still exists concerning the facts themselves and could be responsible, at least in part, for some of the striking differences seen at different times in chronic experiments (see Scheig and Isselbacher, 1965; Isselbacher and Greenberger, 1964; Isselbacher, 1966). It would seem that a systematic study of nutritional and other factors would be absolutely necessary as a basis for the eventual delineation of the biochemical lesion or lesions involved in ethanol-induced fatty liver.

An important aspect of the ethanol effects on fatty acid synthesis relates to the changes in the oxidation-reduction potential in the liver, as seen for example in the ratio of NAD/NADH₂ (see Krebs, 1968). The rate of fatty acid synthesis, as already mentioned, is highly controlled and the balance between NAD and NADH₂ is believed to be an important factor in this control. The effects of ethanol on this balance, by virtue of its own oxidation, is widely appreciated.

A fourth pathogenetic mechanism is an interference with fatty acid oxidation. This could be quantitatively of some considerable importance since the fatty acid oxidation is a major source of energy for hepatic metabolic needs. This oxidation goes on predominantly or exclusively in mitochondria by the classic process of β -oxidation. Coenzyme A and riboflavin play essential roles in this pathway.

One problem prominent in all cells and organelles in regard to lipid metabolism concerns the basic water insolubility of many lipid components and the need for transport in an aqueous medium. Although liver mitochondria are major sites for fatty acid oxidation, they take up fatty acids poorly. To circumvent this problem, the fatty acids, as their CoA derivatives, combine with carnitine to produce a product that is now much more accessible to mitochondrial sites of fatty acid oxidation (see Fritz, 1963). An interference with this metabolic activity might well be a factor in the pathogenesis of some forms of fatty liver.

General Considerations

It is evident from the above discussion that a fundamental aspect in any mechanism of fatty liver development is a change in rate of some reaction or reactions. Therefore, it is

obvious that no explanation is acceptable until it can be shown that the rates of the reactions involved are sufficient to account for the rate of accumulation of TG in the liver under the conditions used. If the rate is sufficiently rapid normally, e.g. supply of FAA in the fasted animal, then relatively slight changes in the rate could have striking quantitative effects very rapidly. If the rate is slow then measurement of the rate of TG accumulation must be compatible with the change in rate of the affected pathway. Such quantitative considerations are essential to any discussion of mechanism but unfortunately are sometimes overlooked or not presented.

It is also not unlikely that an agent such as ethanol may have more than one site of interaction in FA and TG metabolism in the liver. This together with the considerations already presented relating to alternate pathways, makes it almost mandatory that experiments in the genesis of alcoholic fatty liver and liver damage be very highly controlled with respect to amount and composition of the diet, as well as with respect to other physiologic parameters such as hormones that are known to influence the relative balance between the different metabolic pathways in the liver. The uncertainties and contradictions that now confuse the picture might well be lessened if adequate attention were always given to controlling as many of the variables as possible. Hopefully, a clearer need for this may be one of the important consequences of this conference.

HEPATIC NECROSIS

The genesis of cell death in the liver (or in other organs) remains one of the major challenges to research workers in experimental pathology today. It is generally agreed by most pathologists, expert in liver disease, that loss of viability with subsequent necrosis is one of the key pathologic processes leading to chronic liver disease, especially cirrhosis. Despite this almost universal recognition of its importance, we have little insight into its pathogenesis and control. Although a wide variety of toxic agents, viruses and other deleterious hazards in the cell's environment, such as anoxia or ischemia, are known to induce hepatic cell death, no rational pathogenetic mechanisms at cellular or subcellular levels are known.

I would like to review here briefly some of the facets that are known, and to present a working hypothesis for at least one form of cell death. The subject of necrosis or hepatic cell necrosis has been reviewed in the last several years. These reviews are valuable sources of information (Judah, Ahmed and McLean, 1964; McLean, McLean and Judah, 1965; Trump and Ericsson, 1965; Magee, 1966; Judah, 1969; Smuckler and Arcasoy, 1969; Farber, 1963 and 1971(a), 1971(b); Slater, 1972).

Cell death has been considered for a long time to be a "degradative", "degenerative", or "retrograde" phenomenon in which the essential biochemical lesion is some inhibitory influence on one of the many essential metabolic processes in the cell. Given this philosophic approach, it is natural that pathologists and biochemists interested in this process have assumed axiomatically that the cell will die whenever one of many different metabolic pathways are inhibited. In other words, cell death is simply "a running down" of the cell's metabolic activity as a result of the interference with one or more of its major functions.

Studies during the past 10 years or so have disproved much of this and have pointed out clearly that one can interfere with many of the most "basic" properties of the cell without inducing cell death. The strong inference from these studies is that the metabolic mechanisms for cell death are relatively few and are probably quite selective and specific.

Although we have yet to pinpoint *any* single mechanism, the knowledge to date has, nevertheless, been essential for laying the groundwork for the next phase to come: what are the targets for cell death and how are they perturbed? Since unfortunately this knowledge is still not general, I would like to summarize it briefly.

It is now clear that almost every cell, except for a few highly specialized ones like the mammalian erythrocyte with no nucleus or the avian erythrocyte with an inactive nucleus, continually synthesizes RNA needed for the production of its selective proteins both for internal operation of the cell and for the synthesis of any secreted protein. Thus, the transcription — translation apparatus is one of the active metabolic operations in most cells. One would have thought that interference with the function of this metabolic pathway might cause irreversible damage to most cells, especially if the damage persists for hours or a few days. The evidence clearly indicates that such is not the case. Inhibition of RNA synthesis, even up to levels approaching 100 per cent, does not induce liver cell death (J. Farber, 1972; Shinozuka, 1972; E. Farber, 1971). Similarly, severe inhibition of protein synthesis by a variety of agents, even for many hours or a few days, does not lead to irreversible cell damage and necrosis (E. Farber, 1972). It must be emphasized that interference with transcription or translation does cause obvious cellular alterations, including irreversible damage to the bulk of the cytoplasmic ribosomes. However, somehow the essential features of cell integrity remain intact and sufficiently functional to maintain the viability of the cell.

Although interference with DNA synthesis is often considered to be a lethal biochemical lesion, this seems to be true only in certain cell systems. These do not include the liver where one can virtually completely interrupt DNA replication for hours or days without compromising the integrity of the hepatocyte (Schwartz *et al.*, 1965; Farber and Baserga, 1969; Verbin, Sullivan and Farber, 1969; Verbin, Goldblatt and Farber, 1969; Verbin *et al.*, 1971). Thus, interference with cell replication and the synthesis of DNA, RNA or protein cannot be implicated at present in the genesis of liver cell death and necrosis.

The interference with the generation of energy and associated changes in mitochondria have been favorite sites of study in the analysis of cell death. Somehow, it is tacitly assumed that in this aspect of the cell lies the key to cell integrity. In fact, mitochondrial damage was discovered in one of the earliest studies in liver cell death and necrosis (Christie and Judah, 1954). This classical study can be said to have opened up the new and modern phase of study of liver cell death. However, as with so many other leads, it was subsequently shown quite conclusively that the mitochondrial changes were late phenomena and could be accounted for easily as secondary changes *due to* cell death rather than primary causative factors (see Recknagel, 1967).

Also, studies with ethionine and other models have shown that the liver can withstand a very marked (decreases of 80-90%) deficiency of ATP for days without leading to loss of viability (see Farber, 1973(b)). Even though the disturbances in liver ATP concentration produce severe metabolic and structural alterations in the hepatocytes, somehow the changes do not compromise those key sites that are necessary for maintaining hepatocyte integrity (e.g. see Shinozuka *et al.*, 1970, 1971 (a) and (b)).

Lysosomes and the activation and release of their lytic enzymes have been periodically implicated in the genesis of liver cell death (Magee, 1966; Farber, 1963). Like mitochondrial changes in toxic liver injury, the lysosomal alterations appear to be "after the fact" and not primary or major factors in the induction of liver cell death.

Shifts in ion balance have been discussed periodically in relation to the development of hepatocyte necrosis. Clearly, major shifts in Ca^{++} are known to occur *after* the cells

die and these must obviously be excluded in any discussion of pathogenesis. However, there are several studies that are relevant to the early inductive phases of cell death. On the negative side, a large loss of K^+ and an increase in Na^+ in the liver accompanies changes in ATP induced by ethionine (Judah *et al.*, 1966; Christie and Judah, 1968; Okazaki, Shull and Farber, 1968; Vogt and Farber, 1970). Despite these changes, indicative of some interference with the ion exchange of the membrane, the cells do not undergo cell death. Ca^{++} changes do not accompany the changes in Na^+ and K^+ ions.

On the positive side, Ca^{++} changes are known to occur early after the administration of CCl_4 (Thiers, Reynolds and Vallee, 1960) and are believed to play an important role in the pathogenesis of cell death (see Judah, Ahmed and McLean, 1964). Supporting this view are the results of several studies by Gallagher, Judah and Rees and their colleagues (Gallagher *et al.*, 1956; Judah and Rees, 1959; Gallagher, 1961; Rees, Sinha and Spector, 1961; Rees and Spector, 1961) on the protective effects of phenergan and related pharmacologically active compounds against cell death induced by some hepatotoxins. All these studies tend to implicate the plasma membrane and its role in maintaining the proper Ca^{++} balance in the development of irreversible hepatocyte damage. If the plasma membrane is truly the major target for the genesis of cell death, the effects again must be quite specific, since the functional changes accompanying ATP deficiency appear to be affecting some non-vital component of the membrane and no cell death ensues. The development of a whole new technology relating to the plasma membrane now makes available a new approach to the question: is the cell membrane *the* major or only target in the pathogenesis of liver cell death?

In relation to the problem as posed at the beginning of this section, is cell death a passive or an active process, I would like to review briefly some recent work and to present a new working hypothesis. The basis for this formulation began with studies of the effects of inhibitors of DNA synthesis on the intestinal mucosa. It is known that DNA replication can be inhibited either by inhibitors of DNA synthesis *per se* or by inhibitors of protein synthesis. The synthesis of DNA is closely geared to the synthesis of protein, probably histones and many other proteins involved in the mechanism of replication and organization of DNA. The administration of inhibitors of DNA synthesis alone, such as cytosine arabinoside (ara-C) or hydroxyurea regularly induces cell death in the intestinal mucosa while inhibitors of DNA and protein synthesis, such as cycloheximide and tenuazonic acid do not (Lieberman, *et al.*, 1970; Farber, Verbin and Lieberman, 1971). This rather surprising observation led to further studies showing that the two inhibitors of protein synthesis would protect the mucosa against cell death induced not only by ara-C or hydroxyurea but also by X-irradiation or by nitrogen mustard. Furthermore, the inhibitors could be given *after* the initial biochemical lesion induced by each of the agents (30 or 45 minutes after) and still prevent cell death. These observations are consistent with several similar ones made with various cells in culture and with bacteria on "thymineless death" or unbalanced growth (see Lieberman, *et al.*, 1970, Farber, Verbin and Lieberman, 1971; Lieberman, 1972 for references).

All these findings lead us to postulate that cell death, at least in some cells under some conditions, may not be a passive consequence of some key biochemical lesion but rather a more active process, perhaps akin to enzyme induction. According to this view, the irreversible damage to the cell might be due to the overproduction of some enzyme or other protein that occurs as a response to the initial damaging event and that cell death is more akin to "cell suicide" rather than to "cell homicide" (Farber, Verbin and Lieberman, 1971).

More recently, Drs. Popp and Shinozuka have been testing this hypothesis in acute liver cell injury induced by two agents — α -naphthylisothiocyanate (ANIT) and CCl_4 . They first studied ANIT and confirmed that this agent induced an acute necrosis of the biliary epithelium, especially of the interhepatic ducts and ductules (Ungar *et al.*, 1962). This effect was completely prevented by the administration of cycloheximide. This probably explains some of the findings of Plaa and his associates (Redmond, Witschi and Plaa, 1971) on the protective effects of cycloheximide upon the biochemical changes in liver function induced by ANIT.

More recently, Drs. Popp and Shinozuka have found that cycloheximide treatment is highly protective against cell death and necrosis and against fatty change but not against the induction of balloon cells by CCl_4 . These observations are compatible with the thesis that there are at least three lesions induced by CCl_4 and perhaps other hepatotoxic compounds — fatty liver, necrosis and balloon cells. Previous work in our laboratory clearly showed that cycloheximide has no significant effect on the activation of CCl_4 or on the binding of labeled CCl_4 to lipid and protein (Farber, Liang and Shinozuka, 1971). Thus, the effect does not seem to be simply one of preventing CCl_4 from being activated. Rather, it looks more as if cycloheximide is doing something beyond the initial generation of an active derivative.

Thus, we feel that we should begin to rethink the process of hepatic cell death and necrosis from at least two points of view — the search for a specific lesion, possibly on the plasma membrane, that might be responsible for the loss of viability, and the possible role of protein synthesis such as in enzyme or protein induction or the role of some highly labile protein in the active generation of a lesion directly leading to cell death. Basic to such studies is the need for more analyzable models of cell death and necrosis. The currently available models leave much to be desired. We need a model in which a pulse of a necrogenic stimulus or agent can be given lasting only as long as is required to produce irreversible cell damage. Also, we must search for new ways to reverse the process at different times in order to begin to analyze the sequence of events (Farber, 1971 (a); 1972). The development of such models might well allow a new concerted attack on this fundamental problem in liver pathology with the latest molecular and biological tools. Such models could give new insight into how to study genesis of liver cell death in alcoholism and its possible modulation.

ENZYME INDUCTION

The induction of enzymes in liver, as well as in other tissues, by a wide variety of drugs, toxic agents and other environmental alterations is now well recognized, since its initial discovery by Conney, Miller and Miller in 1956. This phenomenon has been shown to be important in the response to many drugs and toxic agents. Rubin, Hutterer and Lieber (1968) first reported enzyme induction in liver by ethanol and subsequently many others have reported a similar phenomenon (Rubin and Lieber, 1972).

We have only begun to explore the full implications of this important response pattern in the liver. However, it is already known to be involved in several pathologic systems in the liver. Ethanol intoxication is only one example. In liver carcinogenesis, enzyme induction plays a major role as a site for either increasing or decreasing the generation of derivatives of several carcinogens. These derivatives may be more or less potent as carcinogens depending upon the drug and the condition. Again, in exposure to such environmental hazards as DDT or other similar compounds, enzyme induction may well play a role in determining the long term consequences to the liver or other organs.

I would like to present to you an interesting variant of this phenomenon which could play a role in the removal of lipid that has accumulated during an acute episode or perhaps at some phase during chronic liver disease. My colleague, Dr. Shinozuka, was studying how the liver removed the large lipid collections, induced in this instance by ethionine (Shinozuka, Lombardi and Farber, 1971 (b)). The lipid droplets become encompassed by large masses of smooth endoplasmic reticulum which obviously are breaking up the large lipid mass. This continues until virtually all the lipid is broken up and reappears as small lipid droplets, liposomes, in the endoplasmic reticulum, indistinguishable from the liposomes that appear during the inductive phase. Bar-On *et al.* (1972) have shown that in a comparable model, the accumulated triglyceride is broken down to fatty acids and that the fatty acids are reutilized for the synthesis of new triglyceride for export. It is highly probable that the induction of some lipolytic enzyme(s) is occurring in association with the masses of smooth reticulum. This raises an interesting question concerning proliferated smooth reticulum in general, since masses of these membranes are so commonly seen in many chronic diseases. Could they be playing some special role in attempted repair of cell damage?

CIRRHOSIS

The pathogenesis of cirrhosis remains one of the problems of highest priority in the study of liver disease. This is particularly important in alcoholism. Even the major outlines of the pathogenetic mechanisms remain very much clouded. In my view, even the question of the possible relationship between chronic fatty liver and cirrhosis remains uncertain. Although the tendency today is to consider the transformation from fatty liver to cirrhosis as unlikely, I cannot convince myself that this tentative conclusion rests on a sound basis in fact (see McLaren, Bitar and Nassar, 1972). In humans, the documentation is at best incomplete and sporadic. In experimental models, the fact that fatty liver of long duration need not lead to cirrhosis, as in chronic orotic acid feeding (Sidransky and Verney, 1965) does not prove that some forms of fatty liver do not develop into cirrhosis.

The importance of hepatic cell necrosis in the genesis of cirrhosis remains the central dominant hypothesis and seems to have ample documentation from both clinical and experimental studies. This is certainly the most accepted hypothesis today for alcoholic cirrhosis. The genesis of liver cell death, as already discussed previously, therefore, remains one of the most pressing needs in the study of all forms of chronic liver disease including that seen in alcoholism.

Another facet that is obviously important in cirrhosis is liver cell regeneration. I personally find this the most confusing aspect of all the many in cirrhosis. The patterns of regeneration, the production of nodular regenerative hyperplasia and the clear-cut distinction between regeneration of liver cells in the liver disorganized by fibrosis and true regenerative or hyperplastic nodules remain, in my view, areas in great need of clarification. I do not see how this can come simply by looking at more and more livers with cirrhosis with an exclusive morphologic approach. As I see it, we must develop new markers for different types of regenerative liver cell populations and new ways to study the evolution of these cell populations.

Let me illustrate this with an example from our own work. We have been studying the histogenesis of liver cancer in animals using various chemicals as the carcinogenic stimuli (Farber, 1973(c), 1973(d); Farber *et al.*, 1973). During the development of liver

cancer, new discrete identifiable populations of liver parenchymal cells appear. These grow with time into discrete gross nodules. If the carcinogen is removed, the majority of nodules seem to disappear. These we call the regenerative nodules. Some persist and these we call for convenience hyperplastic nodules. The latter can be identified as one site of origin of liver cancer.

The so-called regression of the regenerative nodules has been puzzling us for years. During the past year, we have been looking at this more closely and now find that they do not regress at all. They become transformed into mature looking liver and become integrated into the organization of the liver. Although the hyperplastic nodules are histologically and biochemically indistinguishable from the regenerative nodules, they do not seem to undergo transformation to mature liver, at least not so easily. I believe many of them do but only very slowly over many months.

These studies, which are still in progress, point to an important aspect of regeneration in chronic liver disease, possibly in cirrhosis, namely the arrest of normal maturation and the subsequent release of the arrest with transformation and reorganization to normal liver. Although the significance of the arrest and its subsequent release remains to be determined from a functional point of view, one cannot help wondering how important this might be in understanding, at least some aspects of, cirrhosis. This concern is reinforced greatly by the studies of ⁽¹²⁾Smetana (1972) of the development and life history of liver cirrhosis in rhesus monkeys given low protein — high fat diets. Smetana has described an evolution of regenerative hepatocyte populations in his animals which seems to resemble closely that seen in rats developing liver cancer.

Perhaps the important work of ⁽¹³⁾Lelbach (see chapter I of this volume) on the relation of cirrhosis to levels of intake of alcohol may be pertinent in this context. Although fatty liver appears to be directly related to the level of alcohol consumption from zero level of intake, this is not the case with cirrhosis. With the latter, no cirrhosis was found until a certain range of alcohol consumption was reached. The pathogenetic basis for this interesting observation remains to be studied. However, the possible effects of high alcohol intake on the production of damage to chromosomes and on the induction of new cell populations, similar in principle to some of the changes induced by carcinogens, might be worth exploring.

All of these considerations point to another need in the study of cirrhosis — to identify with suitable markers different cell populations and to follow such populations during the development of cirrhosis. Conceivably, puzzling questions such as regression, repair, relation to liver cancer and functional meaning of different cell populations may well become amenable to scientific scrutiny as a logical development from the naturalist approach of the descriptive morphologist. I am convinced that only by truly *experimenting* with and *manipulating* different phases can one begin to analyze the essential nature of the biology of cirrhosis.

GENERAL CONSIDERATIONS

It is clear from this presentation that our knowledge about the basic pathologic processes intimately related to the development of liver disease and alcoholism is spotty, fragmentary and irregular. Some areas, such as fatty liver, are now on a reasonably sound scientific basis. Others, such as cirrhosis, are still very rudimentary and poorly understood.

We are in great need for new, unorthodox ideas with which one can explore new avenues and can apply new tools. The development of cell biology, molecular biology, biochemistry and even developmental biology has made great strides in the past 15 years. These are still poorly used in the study of most forms of liver disease. This delay in the application of exciting scientific tools and concepts to liver disease stems in major part from the lack of suitable models. Liver disease is typically a spectrum of responses of many different cells. At any moment in time, one is confronted by a paralyzing array of cells in all different stages of response. The scientific study of such a model is virtually impossible under these conditions. The models must be simplified and unified by experimental manipulations if they are to be analyzed scientifically. This realization has somehow not been acknowledged generally and is even resisted in some quarters. The ultimate goal of experimental pathology and medicine must not be the reproduction of highly complex multifactorial disease in animals but rather the simplification of response patterns of disease so that hopefully they can be analyzed scientifically.

It is axiomatic in science that only by reducing the number of variables to minimum approaching one, can one hope to understand the nature and pathogenesis of disease. The molecular biologist can use the most elegant way to do this — the single genetic mutant variable. In my opinion, we must begin to look for similar models — by systematically studying many genetic variants for response patterns to discrete pathologic stimuli. Hopefully, such an approach coupled with a cleaner use of drugs, toxic agents, viruses and nutrition may give the hepatologist of tomorrow the scientific tools to analyze and understand the essential nature of the pathologic tissue responses so prominent in all diseases including “alcoholic” liver pathology.

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Effects of Ethanol and Acetaldehyde on Hepatic Mitochondria

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The metabolism of ethanol interferes with certain hepatic functions related to mitochondria, for example, the oxidation of fatty acids and chylomicrons (Lieber, Lefevre, Spritz, Feinman and DeCarli, 1967), and the activity of the citric acid cycle (Lieber, 1967). However, morphologic alterations of mitochondria during or after acute ethanol intoxication are not present or are at most inconspicuous (Tanikawa, 1968). By contrast the livers of chronic alcoholics display enlarged and misshapen mitochondria, with disoriented cristae and occasional crystalline inclusions (Svoboda, Manning, 1964; Rubin and Lieber, 1972).

It appeared, therefore, that chronic ethanol ingestion produced persistent changes in mitochondria, which were superimposed upon any effects caused solely by the metabolism of ethanol. This concept was supported by the observations that chronic ethanol ingestion led to morphologic changes of mitochondria in skeletal muscle (Song and Rubin, 1972), intestine (Rubin, Rybak, Lindenbaum, Gerson, Walker and Lieber, 1972), and pancreas (Darlé, Ekholm and Edlund, 1970), that is, in organs which do not display any appreciable metabolism of ethanol. Yet the possibility remained that the morphologic alterations of hepatic mitochondria were actually caused by nutritional deficiencies, rather than alcohol, per se. We inquired into this possibility by studying the effects of chronic ethanol ingestion with adequate diets in several species. These experiments demonstrated that ingestion of ethanol with an adequate diet leads to striking morphologic changes in hepatic mitochondria of rats (Iseri, Lieber and Gottlieb, 1966; Rubin, Beattie and Lieber, 1970), sub-human primates (Lieber, DeCarli, Gang, Walker and Rubin, 1972; Rubin and Lieber, 1974), and non-alcoholic volunteers (Rubin, Lieber, 1967; Lieber and Rubin, 1968; Rubin and Lieber, 1968).

MORPHOLOGIC STUDIES

Experiments were carried out in rats, baboons and human volunteers. Rats were fed a nutritionally adequate liquid diet which was supplemented with vitamins, minerals and choline (DeCarli and Lieber, 1967), for 24 days. Pair-fed animals were given the same diet, except that ethanol comprised 36 per cent of total calories as isocaloric replacement of carbohydrate. Baboons were fed a similar liquid diet (Rubin and Lieber, 1974), in which ethanol accounted for 50 per cent of total calories. Volunteers were given ethanol as 36-46 per cent of total calories with an adequate or high protein diet (Rubin and Lieber, 1967; Lieber and Rubin, 1968; Rubin and Lieber, 1968). In animals and man, chronic ethanol ingestion led to enlarged and grotesque mitochondria, with disoriented cristae and occasional matrix densities, or (in man) paracrystalline bodies (Fig. 1). Remnants of degraded mitochondria were noted in areas of focal cytoplasmic degeneration. By contrast, no variations from normal were noted in mitochondria of rats 4-18 hours after a single large dose of ethanol.

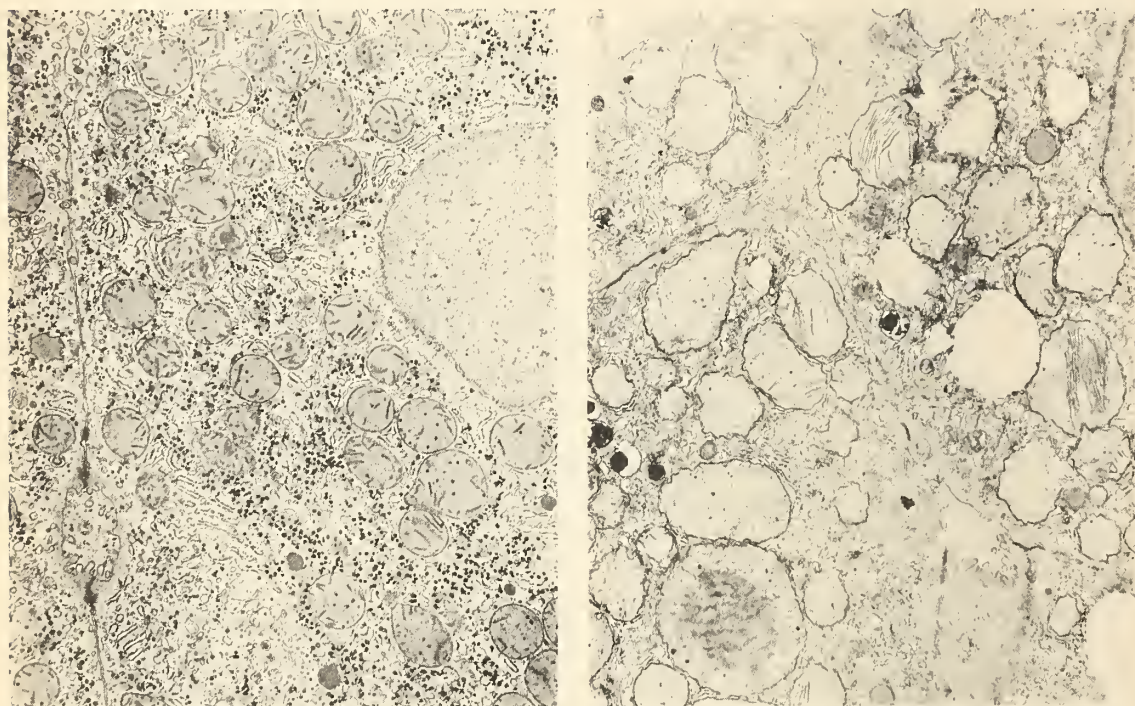


Figure 1. A) Electron micrograph of liver from baboon fed a control high protein diet for 15 months. Mitochondria appear normal. (X 10,800).

B) Electron micrograph of liver from baboon fed ethanol with a high protein diet for 15 months. Mitochondria are enlarged and distorted, and display disoriented cristae. (X 10,800).

MITOCHONDRIAL FUNCTION

Protein Synthesis

Because chronic administration of ethanol apparently resulted in structural changes in mitochondrial membranes by morphologic examination, we studied the effects of ethanol on the biogenesis of mitochondrial membranes in rats.

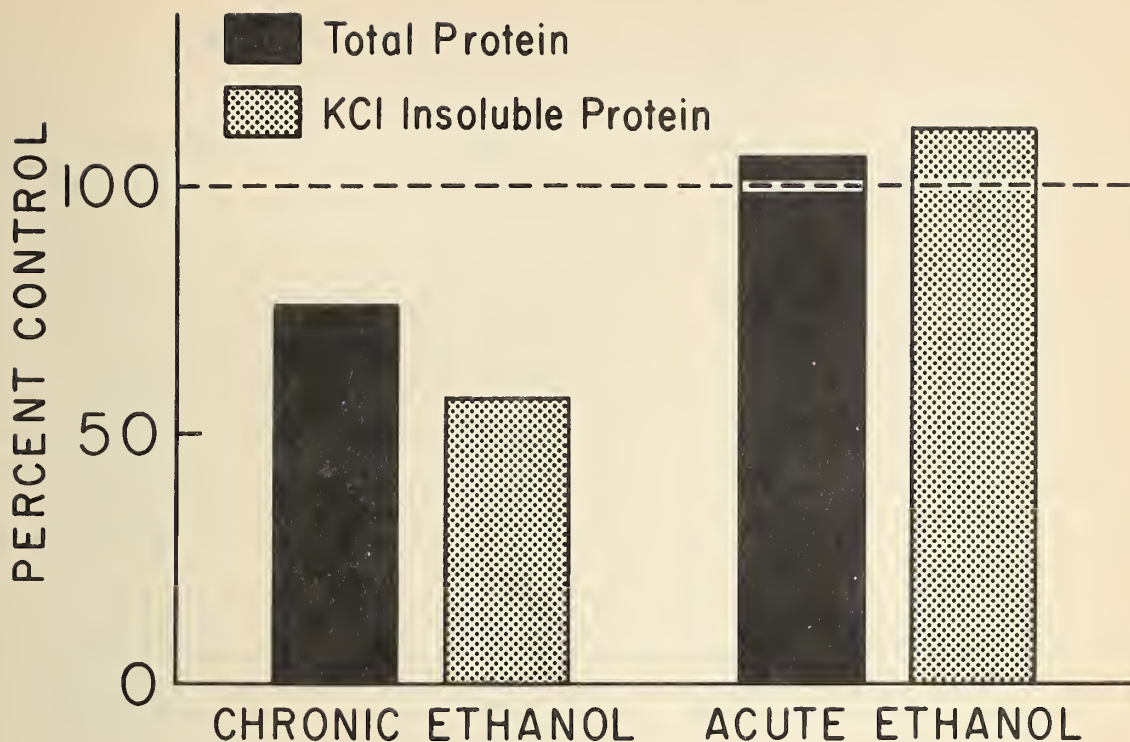


Figure 2. Inhibition of ^{14}C -arginine uptake by hepatic mitochondria *in vivo* in rats fed ethanol chronically.

About 90 per cent of mitochondrial proteins are produced in and transferred from the endoplasmic reticulum, whereas about 10 per cent are synthesized within the mitochondria (Beattie, Pattan and Stuchell, 1970). Mitochondrial protein synthesis is essentially restricted to the supply of certain insoluble proteins of the inner membrane (Beattie, Basford and Koritz, 1967). Total mitochondrial protein synthesis was assessed *in vivo* by measuring the incorporation of ^{14}C -leucine or ^{14}C -arginine into total and KCl insoluble protein of isolated mitochondria (Rubin, Beattie, Toth and Lieber, 1972). Using either labelled leucine or arginine, the incorporation into mitochondria of ethanol treated rats *in vivo* was substantially decreased, compared to pair-fed controls (Fig. 2). We then studied the effect of ethanol on mitochondrial protein synthesis *in vitro* (Rubin, *et al.* 1970), a system in which intramitochondrial protein synthesis is measured independently from the contribution of the endoplasmic reticulum. Ethanol (50-100 mM) inhibited the incorporation of ^{14}C -leucine into mitochondrial protein *in vitro* about 20 per cent (Table I). This effect was not observed in mitochondria from which ethanol has been washed out, a fact which indicates that neither denaturation of protein, nor a solvent effect, explains the inhibition of protein synthesis. Lipid peroxidation played no role, since the presence of antioxidants was also without effect. Thus the presence of ethanol interferes with the synthesis of mitochondrial membranes. Mitochondria isolated from rats fed ethanol chronically but incubated without ethanol, displayed a conspicuous decrease in amino acid incorporation *in vitro*, compared to mitochondria from their pair-fed controls (Table II).

TABLE I

EFFECT OF ETHANOL IN VITRO ON THE INCORPORATION OF ^{14}C -LEUCINE
INTO PROTEIN OF HEPATIC MITOCHONDRIA.^a

Experiment ^b	Protein	Inhibition of C^{14} -leucine Incorporation
	mean c.p.m./mg.	mean %
Standard incubation (40)		
No ethanol	2459 \pm 734	
100 mM ethanol	1959 \pm 432	20.3 ^c
Standard incubation (6)		
No ethanol	2275	
100 mM ethanol	1934	15
50 mM ethanol	2024	11
Addition of various compounds (6)		
No ethanol	1650	
100 mM ethanol	1369	17
Azide (no ethanol)	1240	
Azide + 100 mM ethanol	1017	18
Semicarbazide (no ethanol)	1175	
Semicarbazide + 100 mM ethanol	905	23
DPPD ^d (no ethanol)	1605	
DPPD + 100 mM ethanol	1380	14
Glutathione (no ethanol)	1690	
Glutathione + 100 mM ethanol	1453	14

^aSeparate control incubations were conducted for each experiment in view of the variability among groups of animals.

^bFigures in parentheses indicate the number of control and experimental incubations for each study.

^cS. E. is 3.1; $p < 0.01$ (paired t-test).

^dDPPD, N,N-diphenyl-p-phenylenediamine.

It is reasonable to speculate that the defects in mitochondrial protein synthesis associated with ethanol consumption may be a factor in the production of the structural abnormalities previously demonstrated.

Cytochrome Content

In view of the inhibition of protein synthesis produced by chronic ethanol ingestion, we studied the effect on the cytochromes, which are an integral part of the inner membrane (Rubin, *et al*, 1970) (Table II). The content of cytochrome c, which is easily washed out of the mitochondria, and which is synthesized in the endoplasmic reticulum, was not altered by chronic ethanol feeding. By contrast, the contents of cytochromes b and $a + a_3$ were reduced. In this respect, it is interesting that 3 of the 7 subunits of cytochrome oxidase are synthesized on mitochondrial ribosomes (Mason and Schatz, 1973).

TABLE II

EFFECTS OF CHRONIC ETHANOL INGESTION IN RATS ON INCORPORATION OF ^{14}C -LEUCINE, RESPIRATION AND CYTOCHROME CONTENT IN HEPATIC MITOCHONDRIA.

Experiment ^a	Control Value	Change in ethanol-fed rats	p ^b
	mean \pm S.E.	mean %	
^{14}C -leucine incorporation	996 \pm 86	-29.3 \pm 5.2	< 0.02
Oxygen uptake (Succinate)			
State 4 respiration	18.8 \pm 1.5	-35.3 \pm 7.7	< 0.02
State 3 respiration	47.4 \pm 2.8	-42.8 \pm 7.2	< 0.01
Respiratory control ratio (state 3/state 4)	3.13 \pm 0.6	N.S. ^c	
ADP/0	1.66 ^d	N.S.	
Cytochrome content			
Cytochrome a-a ₃	1.08 \pm 0.09	-20.0 \pm 6.1	< 0.01
Cytochrome b	0.78 \pm 0.02	-15.1 \pm 2.0	< 0.001
Cytochrome c-c ₁	1.75 \pm 0.11	N.S.	

^aUnits are as follows: ^{14}C -leucine incorporation, c.p.m./mg. protein; oxygen uptake, nanoatoms oxygen/mg. protein/min. (succinate substrate); cytochrome concentration, nmoles/mg. protein.

^bPaired t-test.

^cN.S., not significant.

^d3 experiments.

Cytochrome Oxidase and Succinic Dehydrogenase Activity

Mitochondria from ethanol-fed rats showed a 23 per cent decrease in cytochrome oxidase activity (Cederbaum, Lieber, Toth, Beattie and Rubin, 1973) (Fig. 3). A similar extent of inhibition was observed in the homogenate, whether expressed per mg homogenate protein or per g liver. Total hepatic protein was not altered by ethanol feeding. The amount of mitochondrial protein per g liver can be calculated from the activity of cytochrome oxidase in the homogenate and in the mitochondria. Total mitochondrial protein per g liver (Fig. 3), as well as the yield of mitochondria (32-33%) was not affected by chronic ethanol feeding. The activity of succinic dehydrogenase was decreased in mitochondria and in the homogenates obtained from the livers of ethanol-fed rats (Fig. 4). The amount of mitochondrial protein and the yield of mitochondria were again unaltered by ethanol feeding (Fig. 4). Addition of ethanol *in vitro* (up to 700 mM) had no significant effect on the activities of cytochrome oxidase or succinic dehydrogenase.

Substrate Oxidation

Although the change in the redox state produced by ethanol oxidation plays a role in the inhibition of fatty acid oxidation by ethanol, it seemed possible that changes in mitochondria, secondary to prolonged ethanol ingestion, might also be a factor. We therefore measured the ability of isolated mitochondria from chronically treated rats to oxidize fatty acids, in the absence of ethanol, or any change in the redox state (Rubin, *et al*, 1972). In mitochondria from ethanol-fed rats, oxidation of fatty acids was depressed,

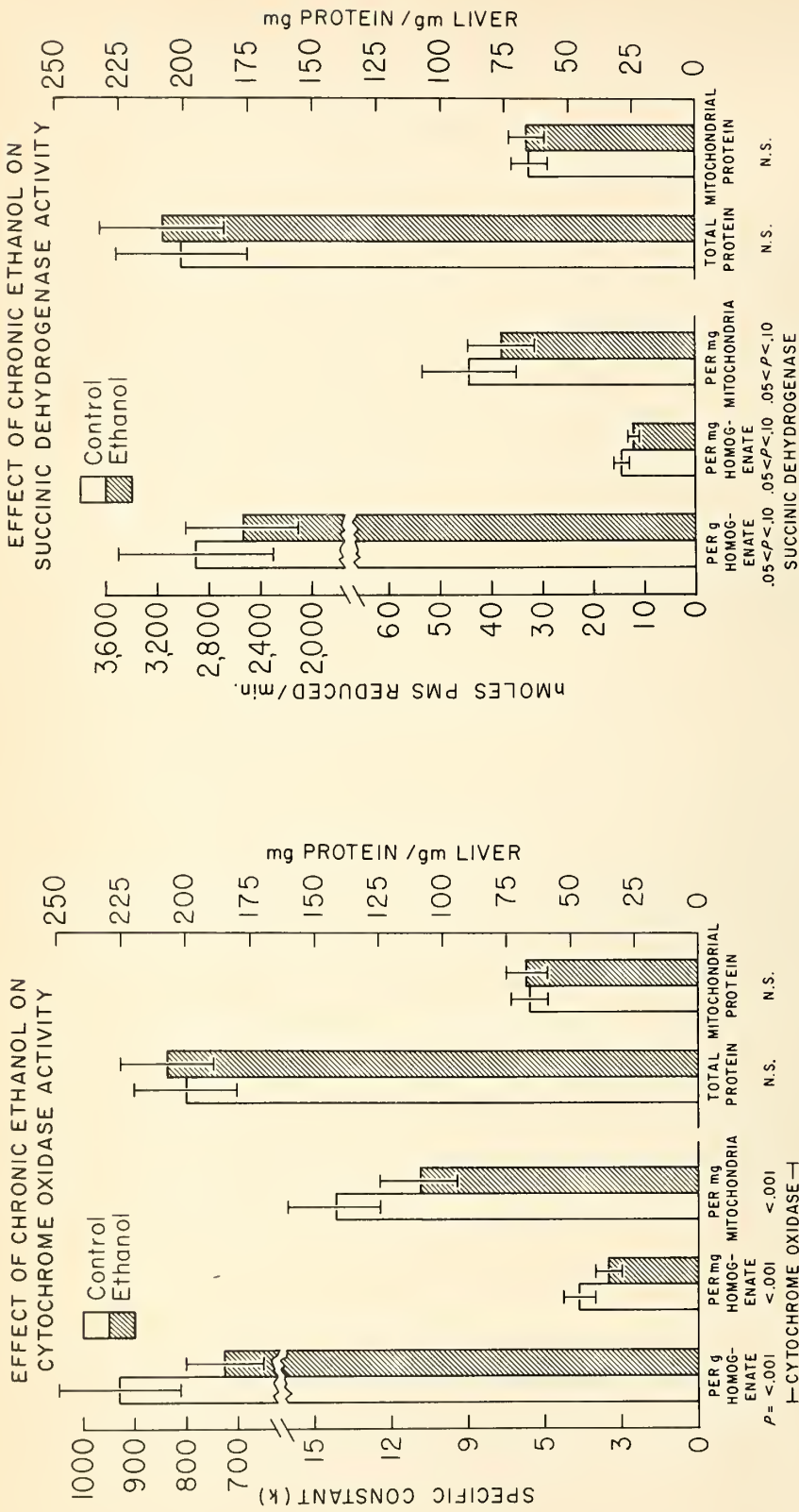


Figure 3. Effect of chronic ethanol on cytochrome oxidase activity — cytochrome oxidase was assayed as described by Wharton and Tzagoloff (1967), following the oxidation of reduced cytochrome C. The first order rate constant was divided by the protein concentration to obtain the specific constant, K .

Figure 4. Effect of chronic ethanol on succinic dehydrogenase activity — succinic dehydrogenase was assayed as described by King (1967), following the reduction of phenazine methosulfate.

whether measured by oxygen consumption (Fig. 5) or CO_2 production, compared to pair-fed controls. The inhibition was similar for all the fatty acids studied (short chain or long chain, saturated or unsaturated). Acute ethanol intoxication had no effect on fatty acid oxidation in isolated mitochondria. The oxidation of succinate was also decreased in mitochondria from ethanol-fed rats, under resting (state 4) or coupled (state 3) conditions (Table II).

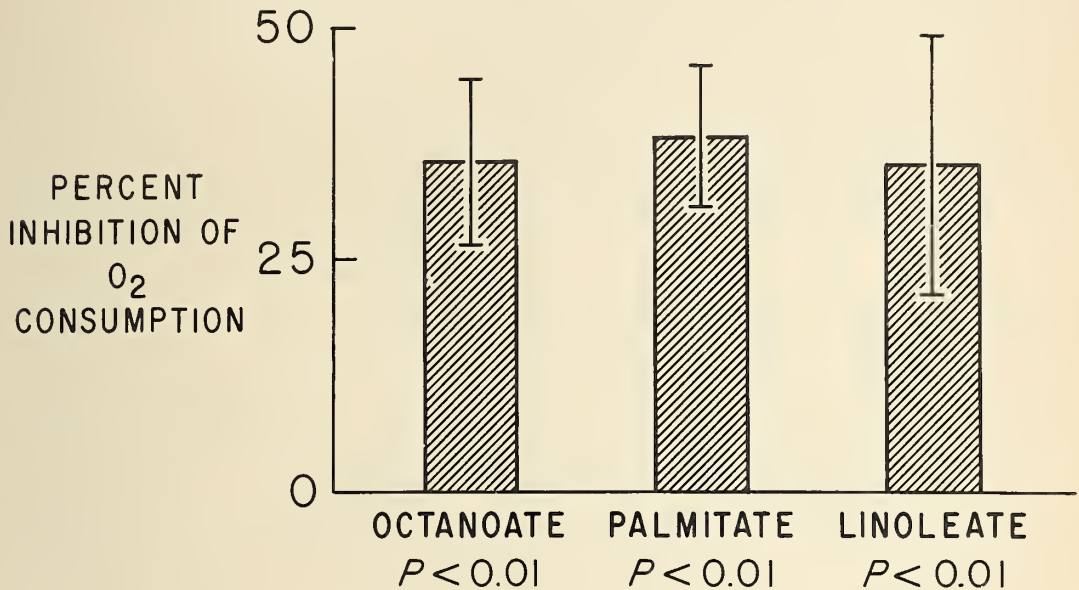


Figure 5. *Inhibition of fatty acid oxidation in mitochondria from rats fed ethanol chronically.*

TRANSPORT AND OXIDATION OF REDUCING EQUIVALENTS INTO MITOCHONDRIA

Chronic administration of ethanol increases the rate of ethanol metabolism in man (Misra, Lefevre, Ishii, Rubin and Lieber, 1971; Kater, Carulli and Iber, 1969) and in rats (Lieber and DeCarli, 1970; Videla and Israel, 1970; Tobon and Mezey, 1971). The factors responsible for this increase are not clearly understood. In general, alcohol dehydrogenase (ADH) activity does not correlate well with ethanol metabolism (Hawkins and Kalant, 1972). Oxidation of ethanol by ADH generates NADH in the cytoplasm; reoxidation of this NADH may then be rate-limiting for the over-all metabolism of ethanol (Vitale, Hegsted, McGrath, Grafle and Zamecheck, 1954; Theorell and Chance, 1951). Since cytoplasmic processes appear insufficient to reoxidize the NADH, mitochondrial oxidation is probably necessary. However, intact mitochondria are impermeable to NADH (Lehninger, 1951). Therefore, several shuttle mechanisms have been proposed for the transport of reducing equivalents into the mitochondria. These include the α -glycerophosphate (α -GP) (Klingenberg and Bücher, 1961), the malate-aspartate (Bücher and Klingenberg, 1958; Borst, 1963), and the fatty acid (Wherret, Orishimo and Nelson, 1969; Grunnet, 1970) shuttles (Fig. 6). Thus the rate of ethanol metabolism might theoretically be affected by several factors: the activity of enzymes responsible for oxidizing ethanol to acetaldehyde,

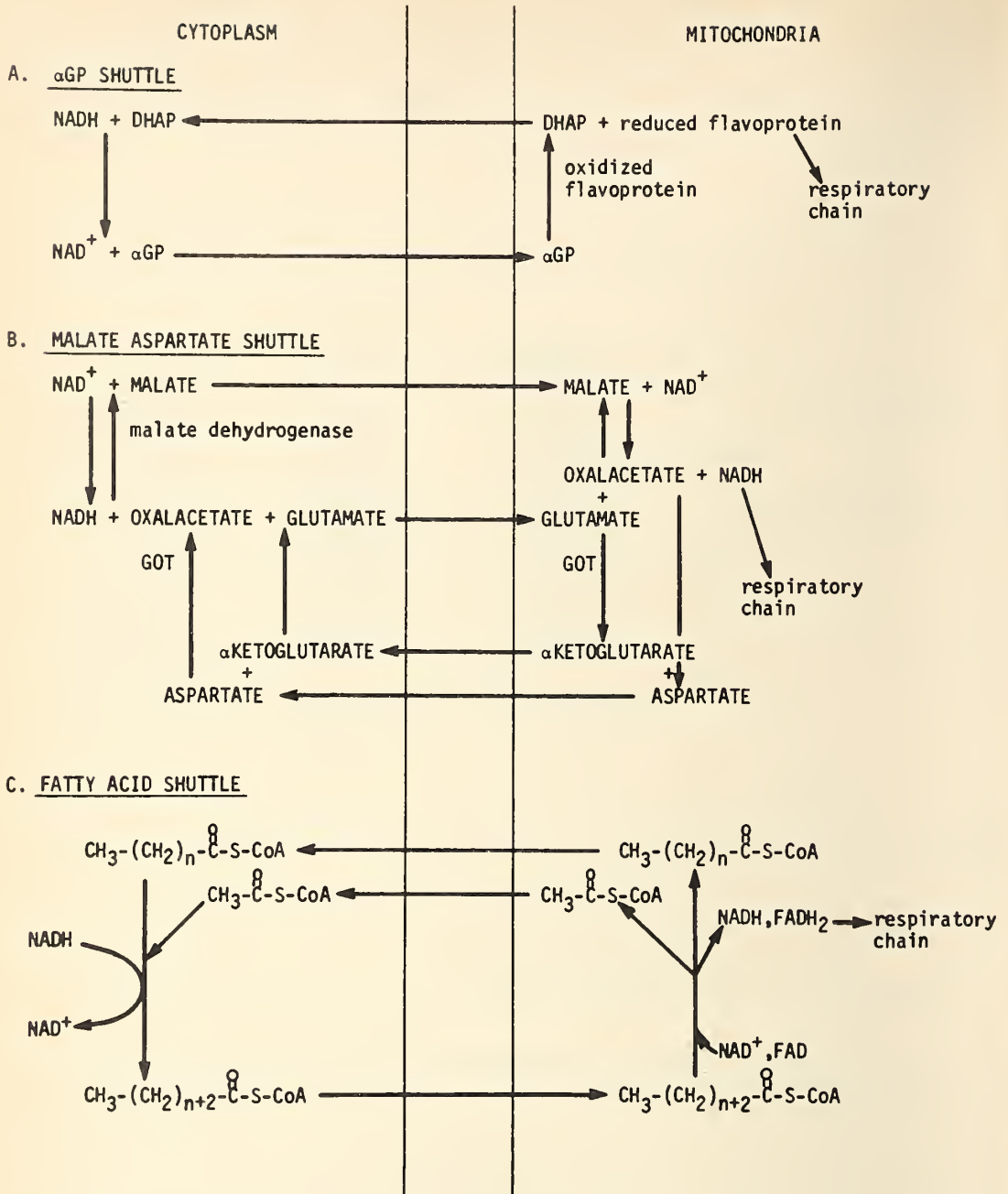


Figure 6. Shuttle mechanisms proposed for the transport of reducing equivalents into the mitochondria — A. α -glycerophosphate shuttle, B. malate-aspartate shuttle, C. fatty acid shuttle.

the activity of systems responsible for transporting reducing equivalents into the mitochondria, and the ability of the mitochondria to oxidize these reducing equivalents.

We therefore examined some of the factors which have been suggested to play a role in the accelerated rate of ethanol metabolism found after chronic ethanol feeding by reconstituting the shuttles *in vitro*.

Reconstitution of the Shuttles

The equilibrium of the ADH reaction favors formation of ethanol and NAD^+ from acetaldehyde and NADH at neutral pH. Therefore the rate of ethanol oxidation is low in the absence of the shuttle system to remove one of the products of the reaction (NADH). Since dissociation and reoxidation of NADH bound to ADH are the rate-limiting steps of the reaction (Theorell and Chance, 1951; Vitale, *et al.*, 1954), the rate of ethanol oxidation in these systems *in vitro* reflects the rate of passage of reducing equivalents into the mitochondria. The shuttles were reconstituted according to methods previously described (Cederbaum, Lieber, Toth, Beattie and Rubin, 1973; Cederbaum, Lieber, Beattie and Rubin, 1973; Lumeng and Davis, 1970; Grunnet, 1970; Lundquist, Thieden and Grunnet, 1970; LaNoue and Williamson, 1971; Rawat and Kuriyama, 1972). The rate of ethanol oxidation in the absence of the shuttle components (fatty acid, α -glycerophosphate, glutamate or α -ketoglutarate) reflects the endogenous rate and is presumably due to direct penetration of NADH into the mitochondria.

It is possible that the oxidized component of the shuttle system may allow ethanol oxidation in the absence of the mitochondria. There is no oxidation of ethanol when the fatty acid shuttle is reconstituted in the absence of mitochondria or when the α -glycerophosphate or malate-aspartate shuttles are reconstituted with α -glycerophosphate or glutamate plus malate, respectively. However, there is considerable oxidation of ethanol in the absence of mitochondria when α -ketoglutarate plus aspartate are used to reconstitute the malate-aspartate shuttle. This system generates oxalacetate, which in the presence of malate dehydrogenase, oxidizes NADH, with a consequent accumulation of malate (and glutamate). The addition of mitochondria increases the rate of ethanol oxidation by about 50 per cent, owing to removal of the malate (and glutamate) and the subsequent generation of additional α -ketoglutarate and aspartate (Fig. 6). Hence there is an artefact when the α -ketoglutarate system is used to reconstitute the malate-aspartate shuttle.

Properties of the Shuttles

Malate-aspartate shuttle. The endogenous rate of ethanol oxidation is about 1-2 nmoles per min. per mg mitochondrial protein, in agreement with the rate of exogenous NADH oxidation by isolated mitochondria (assayed polarographically or spectrophotometrically). When glutamate plus malate were added to reconstitute the malate-aspartate shuttle, ethanol oxidation increased 7 to 9 fold (Table III). Reducing equivalents enter before the rotenone-sensitive site in the respiratory chain, since rotenone and amobarbital inhibit the shuttle to the same extent as cyanide, azide or antimycin (Table III). The inhibition by oligomycin, an inhibitor of energy conservation, and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), an uncoupling agent, indicates that energy is required for efficient operation of the shuttle. This requirement may be necessary for the rapid efflux of aspartate from the mitochondria (LaNoue and Williamson, 1971). For proper operation of the shuttle, malate and glutamate must enter the mitochondria (Fig. 6). Malate can exchange with phosphate, citrate, α -ketoglutarate and other dicarboxylic acids (Robinson, Williams, Haperin and Leznoff, 1971). Iodobenzylmalonic acid, an inhibitor of these malate-exchange reactions (Robinson *et al.*, 1971), inhibited the oxidation of ethanol by the malate-aspartate shuttle. Similarly, avenaciolide, an inhibitor of glutamate transport (McGivan and Chappell, 1970), also inhibited ethanol oxidation (Table III). By

TABLE III

EFFECT OF INHIBITORS ON THE RECONSTITUTED MALATE-ASPARTATE SHUTTLE

Addition	Concentration (mm)	Glutamate +Mitochondria	% Change α -Ketoglutarate -Mitochondria	α -Ketoglutarate +Mitochondria
Rotenone	0.005	-72	-12	-35
Amobarbital	3	-73	+2	-36
Antimycin	0.006	-66	-1	-34
Cyanide	3	-60	-1	-46
Azide	3	-55	+4	-21
Oligomycin	0.008	-44	-13	-27
CCCP	0.003	-43	-5	-25
Iodobenzylmalonate	3.3	-29	-1	--
Iodobenzylmalonate	10	-88	-21	-62
Avenaciolide	0.04	-62	-21	-52
Benzenetricarboxylic acid	20	-4	-1	+2

The endogenous rate was 1.34 ± 0.52 nmoles NADH/min/mg. Addition of glutamate + malate increased this rate to 9.98 ± 1.09 . Addition of a α ketoglutarate + aspartate increased the rate to 10.61 ± 0.55 (about 100 nmoles/min). In the absence of mitochondria, this rate was about 64 nmoles/min.

contrast, 1,2,3-benzenetricarboxylic acid, an inhibitor of malate-citrate exchange (Robinson, *et al.*, 1971), had no effect on ethanol oxidation. Apparently, malate may exchange primarily with α -ketoglutarate, while glutamate may exchange with aspartate, the transport of which shows a glutamate requirement (Azzi, Chappell and Robinson, 1967).

When α -ketoglutarate plus aspartate were added to reconstitute the malate-aspartate shuttle, there was considerable oxidation of ethanol in the presence, and in the absence of mitochondria (Table III). All the inhibitors which caused substantial inhibition of ethanol oxidation when glutamate and malate were used to reconstitute the malate-aspartate shuttle, e.g., rotenone, amobarbital, cyanide, azide, antimycin, CCCP, oligomycin, iodobenzylmalonate or avenaciolide, had little or no effect on ethanol oxidation when α -ketoglutarate plus aspartate were used to reconstitute the shuttle in the absence of mitochondria (Table III). However, these inhibitors almost completely abolished the increase in the rate of ethanol oxidation (about 50%), found when mitochondria were added to the α -ketoglutarate system (Table III). Therefore the rate of ethanol oxidation reflects passage of reducing equivalents into the mitochondria, since inhibitors of mitochondrial function affect only the mitochondrial-dependent systems and not the extramitochondrial system. Furthermore, the mitochondria do not function solely as generators of α -ketoglutarate and aspartate (via mitochondrial transaminase, a matrix enzyme, not linked to the respiratory, coupling or anion transport systems) when glutamate and malate are used to reconstitute the shuttle.

Inhibitors of glutamic-oxalacetic transaminase (GOT) inhibited ethanol oxidation catalyzed by the malate-aspartate shuttle. Hydrazine sulfate (20 mM), cycloserine

(20 mM) and amino-oxyacetic acid (0.2 mM) caused 60-70 per cent inhibition of the rate of ethanol oxidation. At these concentrations, the inhibitors caused a similar extent of inhibition of the activities of the mitochondrial and the cytoplasmic transaminase. Pyridoxal phosphate stimulated the rate of ethanol oxidation (30% at 0.10 mM) at concentrations which stimulate the activities of the transaminase *in vitro*, and which are required for maximal transaminase activity (Stein, Clark and Fortney, 1971).

Fatty acid shuttle. The rate of ethanol oxidation was increased 5-6 fold when the fatty acid shuttle was reconstituted. Shuttle activity was increased to a comparable extent by palmitate, oleate or octanoate (Table IV). The addition of the albumin-bound fatty acids in the presence of ATP, did not alter the permeability of the mitochondria to NADH. The utilization of fatty acid was cyclic since the amount of ethanol oxidized was greater than the amount of fatty acid available, and an increase in the fatty acid concentration (40-200 μ M) did not further increase the amount of ethanol oxidized. Reducing

TABLE IV
RECONSTITUTION OF THE FATTY ACID SHUTTLE

Addition	Concentration (mm)	Activity
None	—	1.16 \pm 0.27
Palmitate	0.1	5.41 \pm 1.20
Oleate	0.1	5.88 \pm 0.63
Octanoate	0.1	5.67 \pm 0.60
		% Change
Rotenone	0.005	-82
Amobarbital	3	-69
Antimycin	0.006	-71
Cyanide	3	-70
Oligomycin	0.008	-83
DNP	0.10	-78
Carnitine	3.3	+24
Isocitrate	3.3	+41
Citrate	3.3	+43
α -Bromopalmitate	0.1	-44

Palmitate was used as the substrate in the inhibitor studies. Activity refers to nmoles of ethanol oxidized/min/mg protein.

equivalents enter the respiratory chain before the rotenone-sensitive site, since the reconstituted shuttle is inhibited by rotenone and amobarbital to the same extent as by cyanide and antimycin (Table IV). Inhibitors of energy-coupling (oligomycin and dinitrophenol) also decreased shuttle activity, suggesting an energy-requirement for the shuttle. This energy-requirement may reflect the fact that the balance between fatty acid elongation and de novo synthesis may be regulated by the ATP content of the mitochondria (Howard, 1968). Omission of ADP and ATP caused 90 per cent inhibition of shuttle activity.

Carnitine increased the activity of the shuttle when palmitate (Table IV) or oleate, but not octanoate, served as the substrate, consistent with the fact that carnitine is required for the transport of long-chain but not short-chain fatty acids (Fritz and Yue, 1965). Citrate has been reported to stimulate the process of chain elongation in hepatic mitochondria (Quagliariello, Landriscina and Coratelli, 1968). Citrate and isocitrate increased the rate of ethanol oxidation catalyzed by the fatty acid shuttle (Table IV). This stimulation by citrate or isocitrate was not prevented by benzenetricarboxylic acid or fluorocitrate. Evidently citrate can stimulate the elongation process in the outer membrane, without undergoing further metabolism or passage through the inner membrane (Cederbaum and Rubin, 1973). In the absence of externally added citrate, endogenous citrate in the mitochondria may stimulate the elongation system of the outer membrane. Preventing efflux of citrate from the inner membrane by adding inhibitors of citrate transport (benzenetricarboxylic acid or iodobenzylmalonic acid) inhibited the fatty acid shuttle (Cederbaum and Rubin, 1973).

The fatty acid shuttle was inhibited by α -bromopalmitate, an inhibitor of carnitine acyltransferase (Chase and Tubbs, 1966), when palmitate (Table IV) or oleate, but not octanoate, served as the substrate. The acyltransferase catalyzes the transfer of long-chain, but not short-chain fatty acids into the mitochondria. α -Bromopalmitate can serve as a substrate for the fatty acid shuttle, although not as efficiently as palmitate.

α -Glycerophosphate (α -GP) Shuttle. Reconstitution of the α -glycerophosphate shuttle increased the rate of ethanol oxidation 6-8 fold. Cyanide caused almost complete inhibition of the shuttle (>90% at 3 mM). Unexpectedly, rotenone also inhibited the shuttle (about 60% at 5 μ M). Therefore the pathway of oxidation of extramitochondrial reducing equivalents by the α -GP shuttle leads partially through NADH dehydrogenase. Rotenone has been reported to inhibit the detritiation of (2)³H-glycerol-3-P in perfused rat liver (Carnicero, Moore and Hoberman, 1972). An alternative explanation may be that rotenone inhibits the efflux of dihydroxyacetone phosphate from the mitochondria. This efflux is required for the cyclic operation of the shuttle (Fig. 6).

Rats were treated with clofibrate (30mg/100g/day, for 14 days), thyroxin (50 μ g/100 g/day, for 7 days) or propylthiouracil (5 mg/100g/day for 21 days), to stimulate or inhibit mitochondrial α -glycerophosphate dehydrogenase activity. Clofibrate and thyroxin increased enzyme activity 3-8 fold, while propylthiouracil decreased activity by 50 percent (Cederbaum, *et al.*, 1973). Despite these changes there were no significant differences in the activity of the reconstituted α -GP shuttle after clofibrate or propylthiouracil treatment, compared to controls. There was an increase in shuttle activity after thyroxin treatment. The activity of mitochondrial α -glycerophosphate dehydrogenase may therefore not be rate-limiting for the α -GP shuttle. Thus the reported increase in ethanol metabolism *in vivo* produced by clofibrate administration (Kähönen, Ylikahri and Hassinen, 1971), does not correlate with an augmented capacity of the α -GP shuttle.

Effect of Chronic Ethanol Treatment on the Transport and Oxidation of Reducing Equivalents

The rate of blood ethanol clearance was increased 44 to 47 per cent in rats chronically fed ethanol (104 ± 15 to 150 ± 20 μ moles ethanol cleared/min/liter, $p < 0.001$). However ADH activity was decreased 20 per cent (13.5 ± 1.18 to 10.63 ± 0.83 nmoles NADH oxidized/min/mg soluble protein, $p < 0.01$).

The endogenous rate for all 3 reconstituted shuttles was the same for mitochondrial preparations from ethanol-fed rats and their controls. Mitochondria from both preparations showed the same rates of exogenous NADH oxidation. Therefore chronic ethanol consumption did not alter mitochondrial permeability toward NADH. However this endogenous rate in rats fed the high-fat diet, with or without ethanol, was about 3-5 fold greater than that observed in mitochondria from rats fed a low-fat diet (see below).

Palmitate, oleate and octanoate were equally effective in reconstituting the fatty acid shuttle in the two types of mitochondrial preparations (Fig. 7). There were no

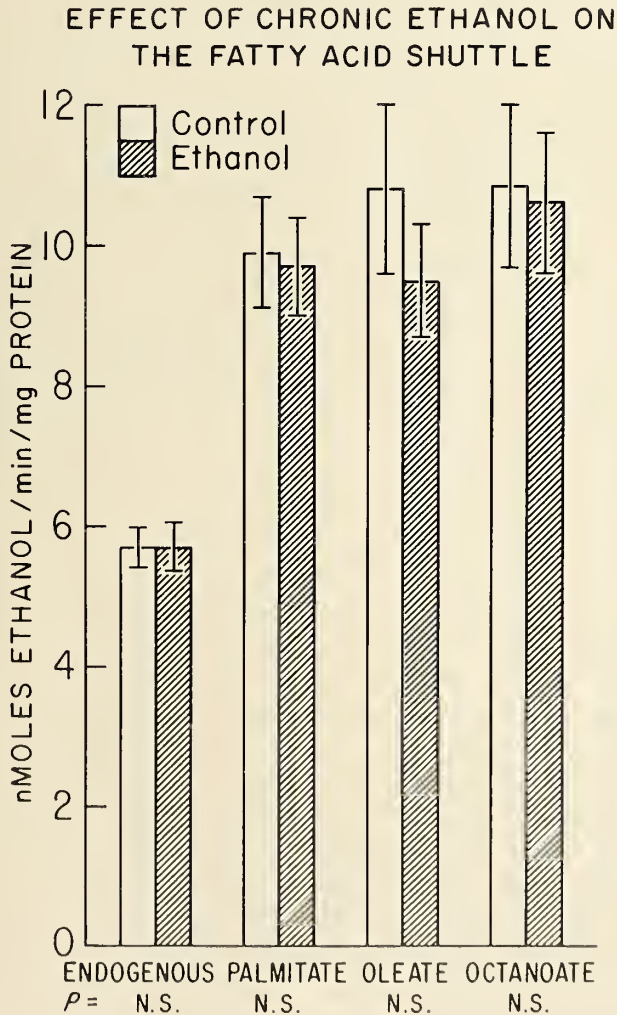


Figure 7. *Effect of chronic ethanol on the fatty acid shuttle — the shuttle was reconstituted with palmitate, oleate or octanoate.*

significant differences in the activity of this reconstituted shuttle between mitochondria from ethanol-fed rats and their pair-fed controls. Several properties of the shuttle, e.g., stimulation by carnitine or by citrate, were similar in both mitochondrial preparations.

Oxidation of α -glycerophosphate by the mitochondria was decreased by ethanol feeding (Cederbaum, Lieber, Toth, *et al.*, 1973). Hepatic cytoplasmic α -glycerophosphate dehydrogenase activity was also decreased in the soluble fraction of ethanol-fed rats. There was a slight decrease in the activity of the α -GP shuttle after ethanol feeding, whether the shuttle was reconstituted with α -glycerophosphate or digydroxyacetone phosphate. This decrease may reflect the lower activity of the mitochondrial α -glycerophosphate dehydrogenase. The mitochondrial enzyme is located on the outer surface of the inner membrane (Klingenberg, 1970). Therefore, α -glycerophosphate need not penetrate the mitochondrial membrane to act as a substrate.

We studied the effect of ethanol feeding on the activity of enzymes which participate in the malate-aspartate shuttle, on the transport of anions which participate in the shuttle, and on the reconstituted shuttle activity itself. Ethanol feeding had no effect on malate dehydrogenase activity (Reed and Mezey, 1972), and the specific activities of mitochondrial and cytoplasmic GOT were unaltered (Figs. 8, 9). The response of GOT to the inhibitors, hydrazine sulfate, cycloserine or amino-oxyacetic acid or to an activator, pyridoxal phosphate, was also unaltered by the ethanol feeding (Figs. 8, 9).

Chronic ethanol consumption had no effect on the activity of the malate-aspartate shuttle reconstituted with either glutamate plus malate (Fig. 10) or α -ketoglutarate plus aspartate (Cederbaum, *et al.*, 1973). Since it was suggested that chronic ethanol feeding may alter mitochondrial permeability toward anions which participate in the malate-aspartate shuttle (Rawat and Kuriyama, 1972), we measured the ability of the mitochondria to take up anions, such as phosphate, malate, glutamate and citrate. Chronic ethanol consumption produced no change in permeability of the mitochondria toward these anions, as measured qualitatively by following the swelling of mitochondria (Chappell, 1968; Chappell and Haarhoff, 1966) in their respective ammonium salts. Both mitochondrial preparations required the additions of the same cofactors for optimal swelling rates, e.g., Pi for malate-induced swelling, Pi plus malate for citrate- or isocitrate-induced swelling (Chappell, 1968; Chappell and Haarhoff, 1966). Addition of ethanol *in vitro* (up to 80 mM) had no effect on the swelling rates. Substitution of NH_4^+ by K^+ reduced the swelling rates of both preparations by 80-95 per cent. Therefore the normally low permeability of control mitochondria to K^+ (Lehninger, Carafoli and Rossi, 1967) was maintained in the mitochondria from ethanol-fed rats.

As additional evidence for the unaltered permeability of mitochondria from ethanol-fed rats to various substrate anions, we studied the influence of anion transport inhibitors on the reconstituted malate-aspartate shuttle. The substrate anions all have distinct carrier systems to be transported into the mitochondria. If ethanol feeding altered permeability to these anions, it might be expected that anion transport inhibitors would not be as effective in mitochondria from ethanol-fed rats as in control mitochondria. Chronic ethanol feeding neither altered the permeability of the mitochondria to the anions, nor affected the response to transport inhibitors such as iodobenzylnalonate (inhibitor of dicarboxylate exchange), avenaciolide (inhibitor of glutamate transport) and benzenetricarboxylic acid (inhibitor of malate-citrate exchange) (Fig. 10). Ethanol treatment apparently did not alter permeability toward adenine nucleotides, since atractyloside, an inhibitor of the adenine nucleotide translocase system, decreased the activity of the malate-aspartate shuttle comparably in both mitochondrial preparations (Fig. 10).

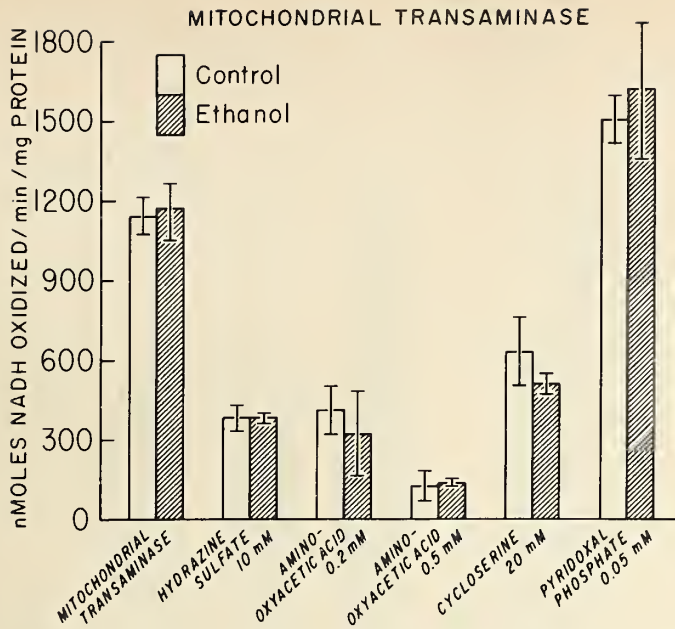


Figure 8. Effect of chronic ethanol on mitochondrial glutamic-oxalacetic transaminase activity — activity was assayed by following the oxidation of NADH as described by Stein *et al*, 1971. To obtain maximal activity of the mitochondrial enzyme, the mitochondria were sonicated for 30s (three 10s bursts) with a Branson LS-25 Sonifier.

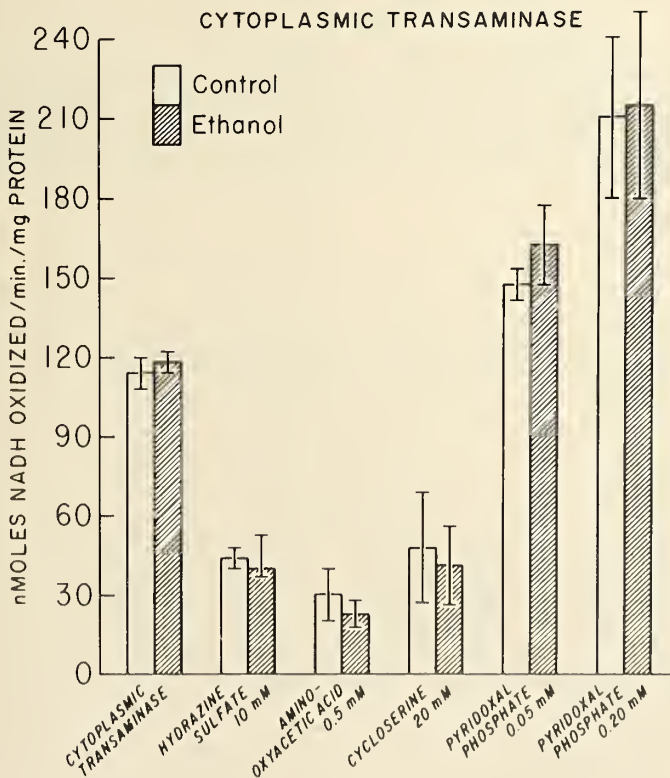


Figure 9. Effect of chronic ethanol on cytoplasmic glutamic oxalacetic transaminase activity.

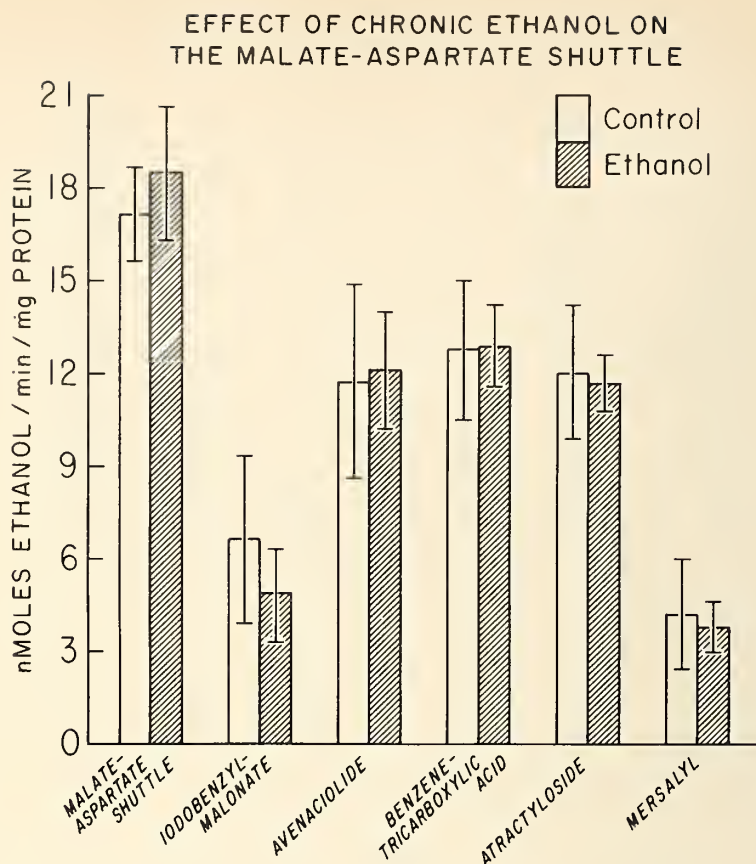


Figure 10. *Effect of chronic ethanol on the malate-aspartate shuttle — the shuttle was reconstituted with glutamate plus malate. The inhibitor concentrations were: 10 mM iodobenzylmalonate, 0.08 mM avenaciolide, 20 mM benzenetricarboxylic acid, 0.04 mM atractyloside, 0.02 mM mersalyl.*

The activities of all three reconstituted shuttles were also unchanged in mitochondria derived from rats given an acute dose of ethanol (5g/kg).

Effect of Dietary Fat

The endogenous rate and the rates of the reconstituted fatty acid and malate-aspartate shuttles were significantly higher in rats fed a high-fat diet (with and without ethanol) than in rats fed a low-fat diet (Fig. 11). A high-fat diet might increase the oxidation of reducing equivalents by isolated mitochondria, either by altering the permeability to NADH or various substrate anions, or by stimulating the activity of an endogenous shuttle, e.g., the fatty acid shuttle, which contains the elongation and β -oxidation systems within the mitochondria, and which may use free fatty acids liberated by phospholipase activity within the mitochondria. If the capacity of the shuttle system is the rate-limiting step in ethanol oxidation, ethanol clearance rates *in vivo* might be expected to be higher in rats fed a high-fat diet than in those fed a low-fat one. However, the rates of blood

ethanol clearance were the same (Fig. 11), suggesting that the capacity of the shuttles to transport reducing equivalents may not be rate-limiting for ethanol oxidation.

EFFECT OF DIETARY FAT ON SHUTTLE ACTIVITY AND ETHANOL CLEARANCE

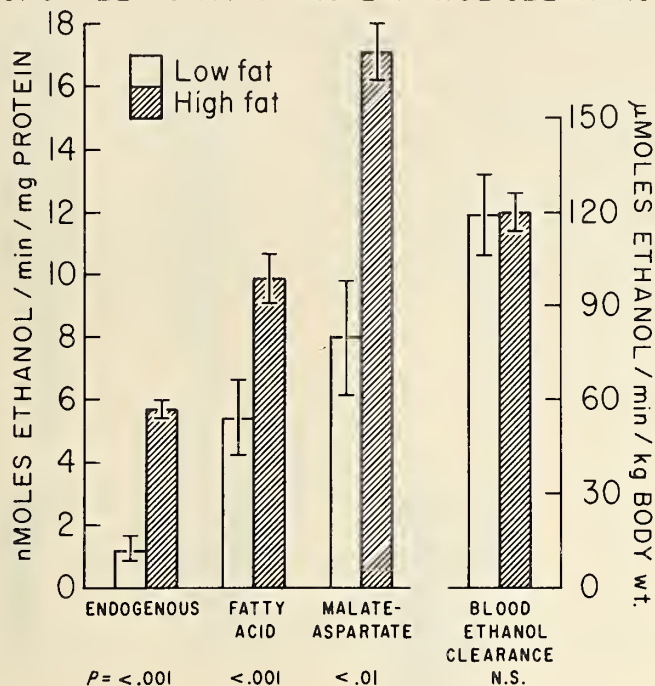


Figure 11. Effect of dietary fat on shuttle activity and ethanol clearance — rats were fed a diet containing either 35 or 9-10% of total calories as fat. Blood ethanol clearance was determined after administering intragastric ethanol (3g/Kg) following an overnight fast. Clearance, calculated as micromoles of ethanol/min/Kg body weight was 105 ± 11 for the high fat group and 104 ± 3 for the low fat group.

EFFECT OF ACETALDEHYDE ON MITOCHONDRIAL FUNCTIONS

After acute ethanol ingestion, acetaldehyde levels in the blood increase. In man, levels of blood acetaldehyde after ethanol ingestion can be as high as 0.2 mM (Majchrowicz, Bercaw, Cole and Gregory, 1967; Truitt and Walsh, 1971). After disulfiram (an aldehyde dehydrogenase inhibitor) administration, levels up to 1 mM (Truitt and Walsh, 1971) have been reported. Since blood acetaldehyde reflects total body concentration, the concentration in liver cells may be considerably higher. The concentration of blood acetaldehyde may also be higher in chronic alcoholics, in view of the accelerated rate of ethanol oxidation (Misra, *et al.*, 1971; Kater, *et al.*, 1969). Most of the acetaldehyde dehydrogenase activity of the liver is in the mitochondrial fraction (Grunnet, 1973; Marjanen, 1972), and is represented by a NAD⁺-dependent dehydrogenase (Smith and Packer, 1972). Acetaldehyde has been found to decrease the oxidation of pyruvate (Kiessling, 1963), acetate and hexanoate (Lindros, 1972) and to stimulate energy-dependent phosphate swelling (Byington and Yeh, 1972). In view of the importance of the mitochondria in oxidation of acetaldehyde and ethanol, we studied the effects of acetaldehyde on mitochondrial functions, including the transport of reducing equivalents.

Oxygen Consumption

Substrates were used which supply electrons to the respiratory chain at the level of NADH dehydrogenase (α -ketoglutarate), at ubiquinone, in the middle of the chain (succinate), and at cytochrome c, near the terminus of the chain (ascorbate). With α -ketoglutarate, 1-3 mM acetaldehyde had little effect on state 4, the resting respiration, whereas state 3 (coupled) respiration was inhibited (Fig. 12). Higher acetaldehyde concentrations inhibited both states of respiration. The greater sensitivity of coupled respiration to acetaldehyde is reflected in the decrease of the respiratory control (state 3/state 4) associated with α -ketoglutarate oxidation. Similar results were obtained with other NAD⁺-dependent substrates, e.g., glutamate and β -hydroxybutyrate. Acetaldehyde also inhibited NADH oxidation by mitochondria rendered permeable to the nucleotide (freeze-thawing). The addition of NAD⁺ to freeze-thawed mitochondria did not prevent

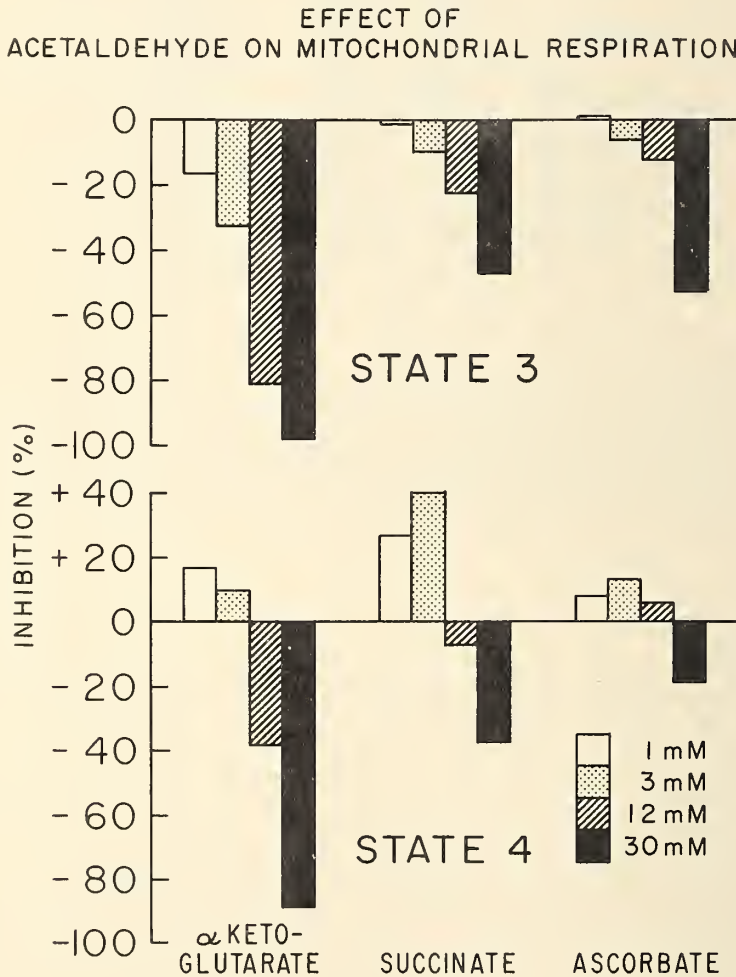


Figure 12. *Effect of acetaldehyde on mitochondrial respiration* — oxygen uptake was assayed polarographically using a Clark oxygen electrode, in the absence (state 4) or presence of 1.5 mM ADP (state 3). Substrate concentrations were: 20 mM α -ketoglutarate, 10 mM succinate, and 5 mM ascorbate plus 0.2 mM tetramethyl-*p*-phenylenediamine.

EFFECT OF ACETALDEHYDE ON OXIDATIVE PHOSPHORYLATION

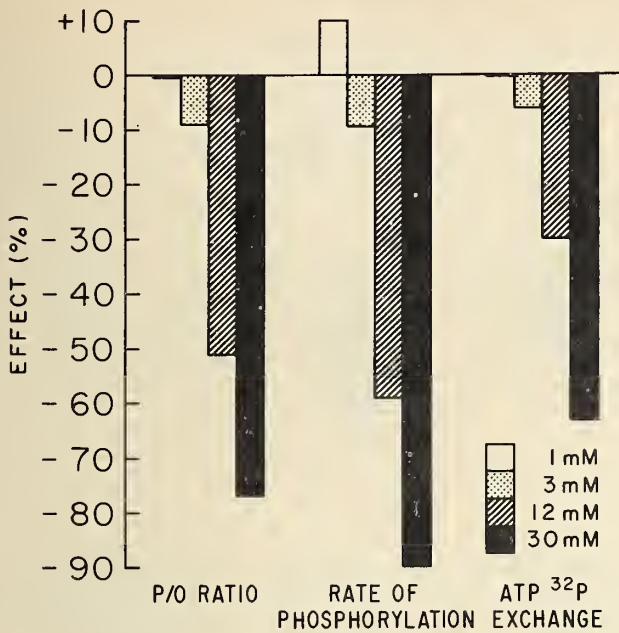


Figure 13. Effect of acetaldehyde on oxidative phosphorylation — the P/O ratio and the rate of phosphorylation were determined with succinate as the substrate. Control values were: P/O ratio, 1.51; rate of phosphorylation 127 nmoles ATP per minute per mg protein; ATP-³²p exchange, 104 nmoles ³²ATP formed per minute per mg protein.

EFFECT OF ACETALDEHYDE ON MITOCHONDRIAL Ca²⁺ UPTAKE

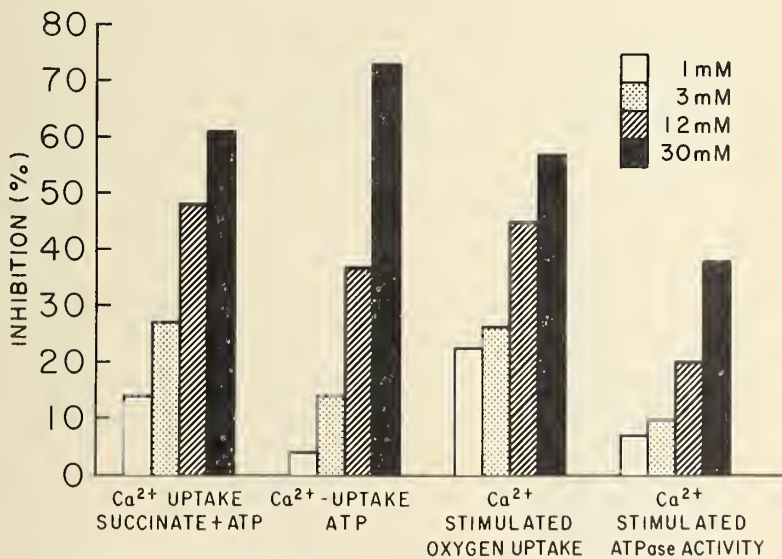


Figure 14. Effect of acetaldehyde on mitochondrial Ca²⁺ uptake — Ca²⁺ uptake was energized by either 10 mM succinate plus 3mM ATP or by 5 mM ATP plus 5 ug anti-mycin. 45 Ca²⁺ was used, along with millipore filtration (0.45μ filters) to remove the mitochondria. Succinate was used as the substrate in the oxygen consumption experiment. Control values were: Ca²⁺ uptake, 399 and 172 nmoles Ca²⁺ per mg protein for the succinate (+ATP) and ATP supported systems, respectively; oxygen uptake, 129 nmoles oxygen per minute per mg protein (rate in the absence of Ca²⁺ = 41); ATPase activity 240 nmoles Pi liberated per minute per mg protein (rate in the absence of Ca²⁺ = 80).

the inhibition of glutamate oxidation by acetaldehyde. Therefore acetaldehyde inhibits the respiratory chain directly, and does not merely compete with the NAD⁺-linked substrates for mitochondrial NAD⁺. With succinate as the substrate, state 4 respiration was stimulated by 1-3 mM acetaldehyde, while inhibition was observed only at higher concentrations (Fig. 12). State 3 respiration was inhibited only at high acetaldehyde concentrations. Ascorbate oxidation was generally insensitive to acetaldehyde, being inhibited only at high concentrations (Fig. 12). The data suggest that an acetaldehyde-sensitive locus is located between NADH dehydrogenase and ubiquinone (the site of entry of electrons from succinate), thereby accounting for the greater inhibition of NAD⁺-linked substrates. Inhibition of succinate or ascorbate oxidation may be due to inhibition of succinic dehydrogenase and cytochrome oxidase by higher concentrations of acetaldehyde. Indeed, 12-30 mM acetaldehyde inhibited the activities of succinic dehydrogenase and cytochrome oxidase (Cederbaum, Lieber and Rubin 1974). By contrast 6-80 mM ethanol had no significant effect on any of these reactions.

Energy Production and Utilization

The respiratory control ratio associated with the oxidation of NAD⁺-linked substrates or succinate was decreased by 1-3 mM acetaldehyde. Uncoupler-stimulated respiration was also less sensitive to acetaldehyde than was ADP-stimulated respiration. Higher concentrations of acetaldehyde (12 mM) inhibited the P/O ratio of oxidative phosphorylation, the rate of phosphorylation and the ATP-³²P exchange reaction, a partial reaction of oxidative phosphorylation (Fig. 13). Acetaldehyde may also play a role in the reported decrease in respiratory control (Banks, Kline and Higgins, 1970) and ATP content (French, 1966) of ethanol-fed rats. The ability of mitochondria to accumulate Ca⁺⁺ is an important energy-utilizing reaction, which may be energized by substrate oxidation or by ATP itself (Lehninger, *et al.*, 1967). Acetaldehyde decreased energy-linked Ca⁺⁺ uptake, when supported by either system (Fig. 14). In addition, acetaldehyde also inhibited stimulation by Ca⁺⁺ of oxygen uptake and of ATPase activity, reactions which are dependent upon the uptake of Ca⁺⁺ (Fig. 14). Ethanol (6-80 mM) had no significant effect on any of these reactions.

There are some indications that acetaldehyde does not appreciably damage the mitochondrial membrane. Part of the acetaldehyde inhibition of Ca⁺⁺ uptake was reversible by washing the mitochondria. Acetaldehyde (1-30 mM) did not stimulate latent ATPase activity nor alter the normally low permeability of the mitochondria to pyridine nucleotides.

Anion Uptake

Lower concentrations of acetaldehyde (1-3 mM) inhibited the uptake of citrate by mitochondria (Fig. 15). Uptake of glutamate and phosphate was inhibited only at higher concentrations of acetaldehyde (> 12 mM). By contrast, uptake of the dicarboxylate malate anion was stimulated by higher acetaldehyde concentrations (Fig. 15). Swelling induced by ammonium citrate was very sensitive to 1-3 mM acetaldehyde, whereas that induced by ammonium phosphate was inhibited only at higher concentrations (12 mM). However, swelling induced by ammonium succinate (substrate for the dicarboxylate carrier) was stimulated by acetaldehyde. These data support those obtained by following

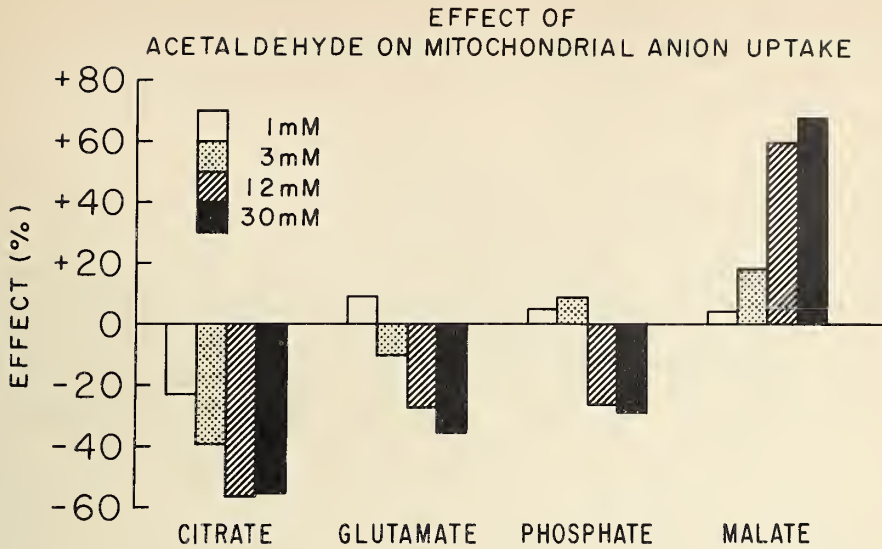


Figure 15. *Effect of acetaldehyde on mitochondrial anion uptake* — anion uptake was assayed by using ^{14}C -labelled anions and rapidly filtering the samples through millipore filters (0.45 μ). Control values of anion uptake (nmoles anion per mg protein) were: citrate, 5.16; glutamate, 6.90; phosphate, 13.7; malate, 11.3.

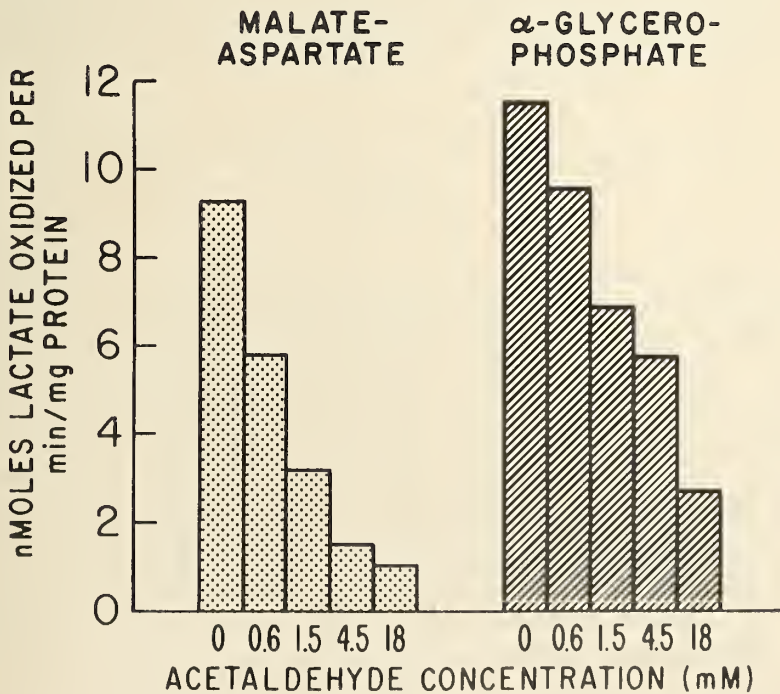


Figure 16. *Effect of acetaldehyde on the malate-aspartate and α -glycerophosphate shuttles* — lactate-LDH was used as the extramitochondrial NADH generating system.

labelled anion uptake. The stimulation of dicarboxylate (malate or succinate) uptake by acetaldehyde may explain the increase in state 4 succinate oxidation by acetaldehyde, since the rate of succinate oxidation is dependent upon the uptake of succinate into the mitochondria (Quagliariello and Palmieri 1968; Luciani, 1970).

Transport and Oxidation of Reducing Equivalents (Shuttles)

Acetaldehyde was a potent inhibitor of all 3 reconstituted shuttles when ethanol-ADH was the NADH-generating system. However, since the equilibrium of the ADH reaction favors ethanol formation from acetaldehyde at neutral pH, using this system may introduce an artefact, thereby increasing the "remaining" ethanol concentration. Indeed, using lactate-lactic dehydrogenase to generate NADH, the addition of acetaldehyde and ADH resulted in the production of ethanol. However using the lactate-lactic dehydrogenase system to generate NADH, acetaldehyde was still a potent inhibitor of shuttle activity (Fig. 16). The extent of inhibition was somewhat less than that found using the ethanol ADH system, and this difference can be accounted for by the reduction of acetaldehyde to ethanol in the latter system. Acetaldehyde (0.6-4.5 mM) had no effect on the activities of GOT, malate dehydrogenase or lactic dehydrogenase, nor did it alter permeability to NADH. Purified α -glycerophosphate dehydrogenase activity was inhibited 14, 23 and 63 per cent by 1.5, 4.5 and 18 mM acetaldehyde, respectively. Acetaldehyde inhibited the extramitochondrial malate-aspartate system (α -ketoglutarate plus aspartate) capable of NADH oxidation much less than the mitochondrial-dependent system reconstituted with glutamate plus malate. The inhibition of the shuttles by acetaldehyde may be related to several factors, e.g., inhibition of the respiratory chain, inhibition of energy production and utilization, inhibition of the transport of anions which participate in the shuttles (glutamate or phosphate) or inhibition of α -glycerophosphate dehydrogenase.

SUMMARY

Mitochondria from ethanol-fed rats display impaired ability to oxidize various substrates, decreased amino acid incorporation into protein, reduced contents of cytochromes b, a and a_3 , and decreased activity of cytochrome oxidase and succinic dehydrogenase. We find no change in mitochondrial yield or mass per g of liver in ethanol-fed rats.

These studies indicate that the acceleration of ethanol oxidation induced by chronic ethanol consumption is not explained by increased capacity of the shuttles for the transport of reducing equivalents into mitochondria. Other factors including pathways unrelated to ADH (MEOS or catalase), extrahepatic oxidation of ethanol, coenzyme availability or altered regulation of mitochondrial functions *in vivo*, should be considered.

The effects of acetaldehyde on mitochondrial function are in many ways similar to those of chronic ethanol administration, e.g., decreased activities of succinic dehydrogenase and cytochrome oxidase, and impaired oxidation of various substrates. Ethanol at concentrations which may be found *in vivo* had no effect on any of these reactions. The inhibition of the reconstituted shuttles, of electron transport and of energy formation and utilization suggests that acetaldehyde may influence the overall metabolism of ethanol.

ACKNOWLEDGEMENTS

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Increased Oxidative Capacity in the Liver Following Chronic Ethanol Administration

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The liver is the principal organ concerned with the metabolism of ethanol (Lundsgaard, 1938; Mirsky and Nelson, 1939). In the cytoplasm of the liver cell, ethanol is first oxidized to acetaldehyde and then to acetate by the NAD-dependent alcohol and aldehyde dehydrogenases respectively. A NAD-dependent oxidation of acetaldehyde has been recently shown to occur also in the mitochondrial compartment (Lindros, Vihma and Forsander, 1972; Grunnet, 1973). The acetate produced in this sequence is further metabolized via the citric acid cycle mainly in extrahepatic tissues (Lundquist, 1962; Lindeneg, Mellemegaard, Fabricius and Lundquist, 1964). As the result of the oxidation of ethanol to acetate, NAD is reduced to NADH; the reducing equivalents produced in the cytosol are transported into the mitochondria by shuttle mechanisms (Lehninger, 1964; Greville, 1969) and reoxidized by the respiratory chain.

We have previously postulated that the rate of mitochondrial oxidation of reducing equivalents, rather than the amount of alcohol dehydrogenase (ADH), is normally the rate-limiting step in the oxidation of ethanol by the liver (Videla and Israel, 1970). Uncouplers of oxidative phosphorylation such as 2,4-dinitrophenol (DNP) or arsenate which are known to increase the rate of mitochondrial oxidation, increase the rate of ethanol metabolism in liver slices (Videla and Israel, 1970), isolated perfused liver (Seiden, Israel and Kalant, 1974) and *in vivo* (Harger and Hulpieu, 1935; Ewing, 1940; Israel, Khanna and Lin, 1970a). This increase in the rate of ethanol metabolism elicited by mitochondrial uncouplers can be blocked by pyrazole (Carter and Isselbacher, 1973) a well known inhibitor of ADH (Lester, Keokosky and Felzenberg, 1968; Goldberg and Rydberg, 1969).

INCREASED OXIDATIVE CAPACITY IN THE LIVER FOLLOWING
CHRONIC ETHANOL ADMINISTRATION

It is a known fact that after prolonged ethanol ingestion the liver shows an enhanced capacity to oxidize ethanol both *in vivo* and *in vitro* (Hawkins, Kalant and Khanna, 1966; Kater, Carulli and Iber, 1969; Videla and Israel, 1970; Lieber and DeCarli, 1970; Ugarte and Valenzuela, 1971; Mezey and Tobon, 1971; Videla, Bernstein and Israel, 1973; Pieper and Skeen, 1973). This enhanced metabolic activity has been shown to occur without concomitant changes in either ADH or catalase activities (Lieber and DeCarli, 1970; Tobon and Mezey, 1971; Mezey, 1972; Videla *et al.*, 1973). It has also been shown that, in this condition, the respiration of the liver tissue is markedly increased in a way that accounts stoichiometrically for all the extra-metabolism of ethanol (Videla *et al.*, 1973; Bernstein, Videla and Israel, 1973; Israel, Videla, Fernandez and Bernstein, submitted for publication). However, mitochondrial uncouplers are not able to further increase either the extra-metabolism of ethanol or the extra-respiration (Videla and Israel, 1970; Videla *et al.*, 1973). The lack of effect of these uncouplers suggests that the mitochondria in the liver of the animals chronically treated with ethanol are respiring at maximal rates either because they are uncoupled or because they are in a respiratory state closer to state 3 of phosphorylation than to state 4 (Chance and Williams, 1956).

We have studied several parameters of mitochondrial function in isolated rat liver mitochondria, from animals chronically treated with ethanol (Videla *et al.*, 1973). Respiratory rates in state 3 and state 4, respiratory control ration (RCR) and DNP-stimulated respiration using either succinate or β -hydroxybutyrate as substrates were not modified by chronic ethanol administration. However, a slight degree of uncoupling in site 1 of phosphorylation, measured as ADP/0 ratio, was observed. A reduction in ADP/0 ratio without change in RCR could conceivably occur if, in the mitochondria of the livers of the animals chronically treated with ethanol, some endogenous substrates enter the respiratory chain at site 2 of phosphorylation. In fact, respiration of these mitochondria in the presence of exogenous ADP but in the absence of added substrates, is significantly higher than that of control mitochondria (unpublished data). No changes were found after chronic treatment with ethanol in either the mitochondrial Mg^{2+} -activated ATPase or the DNP-activated ATPase activities. These data indicate that a mitochondrial uncoupling as measured *in vitro* is not a constant concomitant of chronic ethanol ingestion, in agreement with previous reports by Sardesai and Walt (1969) and Rubin, Beattie and Lieber (1970). However, the possibility of a mitochondrial uncoupling *in situ* in the intact cell cannot be excluded.

It is well known that the rate of oxygen utilization by the mitochondria is regulated by the relative concentrations of adenine nucleotides and inorganic phosphate, generally expressed as the phosphorylation potential (ATP/ADP \times Pi) (Chance and Maitra, 1963; Klingenberg, 1968; 1969). A decrease in the phosphorylation potential leads to an increase in the rate of respiration, until substrate availability, oxygen supply or enzymatic activities become limiting; in this situation a concomitant loss of the activatory effect of uncoupling agents on respiration is likely to occur. Since it appeared that the minimal changes found in isolated mitochondria could not explain the increase in oxygen consumption and the lack of DNP effect in the liver of animals chronically treated with ethanol, our attention was turned to possible changes in the phosphorylation potential of the liver cell.

Animals chronically treated with ethanol showed a significant decrease (about 40%) in the phosphorylation potential in the liver cell; this change was mainly caused by a fall

in the content of ATP and a rise in that of inorganic phosphate (Bernstein *et al.*, 1973). Similar changes in the content of ATP and Pi in the liver of mice and rats chronically treated with ethanol have been reported (French, 1966; Ammon and Estler, 1967; Walker and Gordon, 1970) which would also lead to a reduction in the phosphorylation potential.

The change in the metabolic state of the liver represented by a reduction in the phosphorylation potential could occur if the net rate of ATP utilization exceeds that of its formation so that a new steady state level is reached. An important mechanism that uses ATP is the sodium pump located in the plasma membrane of the cell (Skou, 1965; Albers, 1967; Whittam and Wheeler, 1970). Despite the fact that this system has been estimated to account for about 10% of the total oxygen consumption of the normal liver (Judah and Ahmed, 1964; Van Rossum, 1970), an increased pump activity could conceivably explain the drop in the phosphorylation potential found in the liver following chronic ethanol ingestion.

We have previously reported that in the brain of rats chronically treated with ethanol the (Na+K)-activated ATPase, which constitutes the carrier mechanism for the sodium pump, and the active transport of ^{86}Rb , a functional analog of K^+ (Bernstein and Israel, 1970), are enhanced (Israel, Kalant, LeBlanc, Bernstein and Salazar, 1970b). The increased (Na+K)-ATPase activity in the brain of animals chronically treated with ethanol has been confirmed by other investigators using different species (Sun, 1970; Knox, Perrin and Sen, 1972; Post and Sun, 1973). Similar changes were found to occur in the liver of rats chronically treated with ethanol (Bernstein *et al.*, 1973). The (Na+K)-ATPase and the active transport of ^{86}Rb are markedly increased by 190% and 70% respectively. Further, when liver slices from animals chronically treated with ethanol are incubated with ouabain or in a medium in which sodium is replaced iso-osmotically by sucrose, (conditions which are known to block the sodium pump) (Elshove and Van Rossum, 1963; Skou, 1965; Ruscak and Whittam, 1967), the increased rates of oxygen consumption and ethanol metabolism returned to control values and the activatory effect of DNP was recovered (Bernstein *et al.*, 1973; Israel, Videla, Fernandez and Bernstein, submitted for publication). These results indicate that after chronic administration of ethanol the sodium pump becomes the regulator of cellular metabolism, increasing the oxidative capacity of the liver tissue and offsetting the activatory effect of mitochondrial uncouplers on the rates of oxygen consumption and ethanol metabolism.

Thus, based on available evidence, the following sequence of events is suggested to occur in the liver cell (see Fig. 1): a) chronic ethanol ingestion leads to enhanced utilization of ATP by the (Na+K)-ATPase system, thus b) reducing the phosphorylation potential and c) increasing the rate of oxygen utilisation (and most likely NADH reoxidation) which in turn leads to d) an increase in the metabolism of ethanol and of other substrates.

The increase in the (Na+K)-ATPase activity observed following chronic treatment with ethanol can account for most of the changes found in oxygen consumption and ethanol metabolism. In fact it can be calculated that the increase in the rate of ethanol metabolism elicited by the chronic treatment with ethanol (all of which is sensitive to ouabain) can be accounted for by the ouabain-sensitive extra-oxygen consumption (0.74 mmol ethanol/kg dry wt./min versus 1.15 mmol O_2 /kg dry wt./min). This portion of the oxygen consumption would represent a synthesis of 6.90 mmol ATP/kg dry wt./min assuming a P/O ratio of 3. The increase in (Na+K)-ATPase activity due to treatment amounts to 5.79 mmol Pi/kg dry wt./min, which accounts for more than 80% of the increase in respiration. Similar calculations can be done in order to compare the ouabain-sensitive oxygen consumption with the rate of active transport in the intact cell. How-

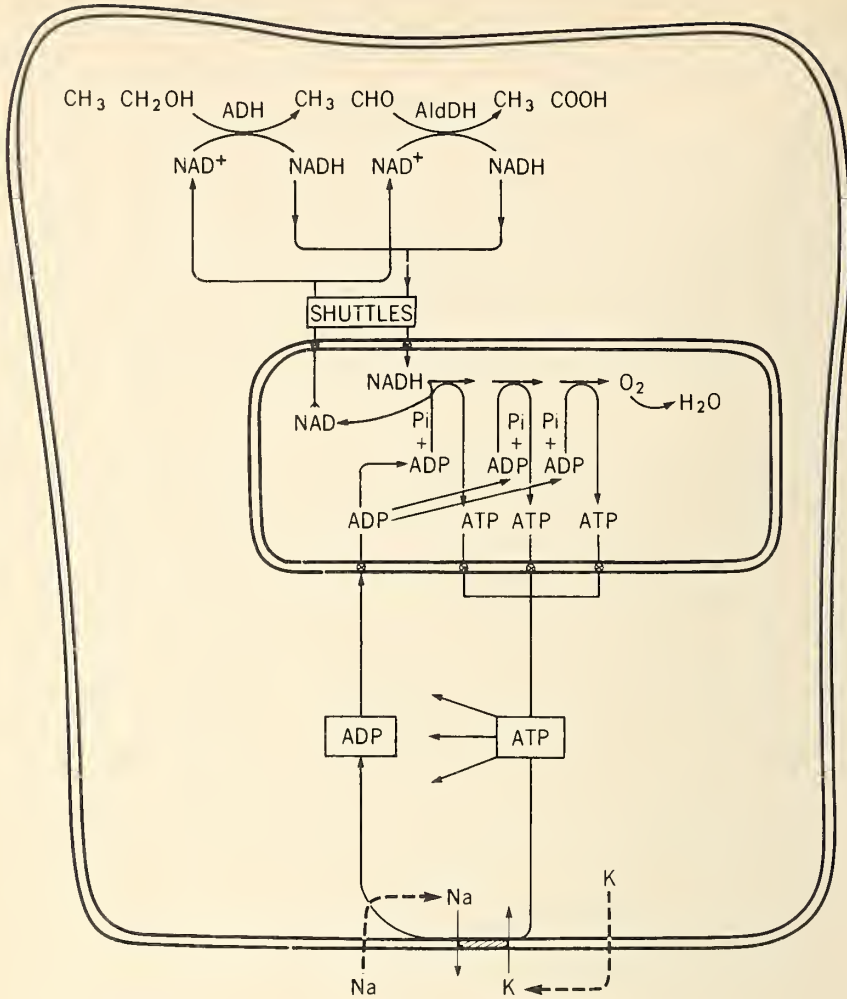


Figure 1. *Schematic representation of the interrelationships between the sodium pump and oxidative metabolism in the liver.*

ever, the K^+/ATP coupling ratio for the liver cell is not known. This ratio has been shown to vary from 2:1 in erythrocytes (Sen and Post, 1964) to 1:1 in brain cortex and 1:3 in seminal vesicles (Whittam, 1964). If a Rb^+/ATP ratio of 1:3 is assumed, the active transport of ^{86}Rb can also account for the increased ouabain-sensitive oxygen consumption observed in the liver of animals chronically treated with ethanol (see Bernstein, Videla and Israel, 1974).

The mechanism(s) by which chronic ethanol administration leads to an increase in the activity of the sodium pump has not been elucidated. The possibilities of an enhanced pump activity as a compensatory mechanism to overcome the inhibitory effect of ethanol on the $(\text{Na}+\text{K})\text{-ATPase}$ system (Israel, 1970; Bernstein *et al.*, 1973) or as the consequence

of hormonal changes accompanying prolonged ethanol ingestion have been recently discussed (Bernstein *et al.*, 1974).

INCREASED OXIDATIVE CAPACITY IN THE LIVER: METABOLIC AND PATHOLOGICAL IMPLICATIONS

The liver has a rather high metabolic rate as judged by its elevated rate of oxygen utilization which can account for up to 25-30% of the total oxygen consumption of the body (Myers and Hickam, 1948; Smythe, Heinemann and Bradley, 1953; Leevy, George, Lesko, Deysine, Abbott and Halligan, 1961; Forsander, 1970). When ethanol is oxidized to acetate, 60-75% of the normal oxygen utilization of the liver is required only for this conversion (Leloir and Muñoz, 1938; Lindros *et al.*, 1972); this would in turn decrease the oxidation of other substrates to a corresponding degree. In fact, carbon dioxide production is drastically depressed by ethanol in the liver of normal animals while the rate of oxygen consumption is not affected (Freinkel, Singer, Gilbert and Anderson, 1962; Lundquist, Tygstrup, Winkler, Mellempgaard and Munck-Petersen, 1962; Majchrowicz and Quastel, 1963; Forsander, Riih  , Salaspuro and M  enp   , 1965; Salaspuro and M  enp   , 1966; Thieden and Lundquist, 1967; Williamson, Scholz, Browning, Thurman and Fukami, 1969; Vendsborg and Schambye, 1970). Studies in our laboratory have shown that the lack of effect of ethanol added *in vitro* on the rate of oxygen consumption by the liver occurred not only in control animals but also in those chronically treated with ethanol, which exhibited a 50% increase in the rate of oxygen utilization (Videla *et al.*, 1973). These findings indicate that in the absence of ethanol the liver of the ethanol treated animals must metabolize other substrates at a faster rate.

We have shown that chronic ethanol administration to rats leads to a 50% increase in the urea production from alanine in the *in vitro* liver-slice system (Israel, Videla, Macdonald and Bernstein, 1973). This finding and the increased urinary nitrogen excretion observed in the chronic situation (Klatskin, 1962; Rodrigo, Antezana and Baraona, 1971) suggest an enhanced amino acid catabolism.

The hypermetabolic state of the liver (increased oxygen and substrate utilization) produced by chronic ethanol ingestion might be of importance in the production of the pathological changes observed in experimental animals and in human alcoholics. If the oxygen utilization of the liver is increased under conditions of normal oxygen supply, a greater oxygen gradient between the hepatocytes located in the periphery and those in the central areas (zone 3 of the hepatic acinus) of the hepatic lobules is likely to occur, thus producing a centrilobular (periacinar) oxygen deficiency. It is important to note that this central area is considered to be the most sensitive to damage due to poor oxygen supply and nutritional deficiency (Rappaport, 1963) and where most of the characteristic histological features of alcoholic hepatitis and cirrhosis appear during heavy ethanol intake (Edmondson, Peters, Reynolds and Kuzma, 1963; Schaffner and Popper, 1970; Jewell, Medline and Medline, 1971). It is known that hypoxic and ischaemic conditions are able to produce drastic changes in the metabolic state of the liver (Bronsnan, Krebs and Williamson, 1970; Ballard, 1971) and to produce centrilobular necrosis (Fraser, Rappaport, Vuylsteke and Colwell, 1951; Rappaport and Lotto, 1951). Further, once necrotic and fibrotic processes develop, the architecture of the hepatic lobules is so deranged that normal supply of blood may be drastically impaired, a condition which will further enhance the prevalent hypoxic state and will also extend the hypoxic area thus producing new necrotic foci.

CONCLUSIONS

Chronic ethanol administration to rats leads to an increased capacity of the liver to metabolize ethanol and an enhanced rate of oxygen utilization. This hypermetabolic state is produced by an increase in the activity of the sodium pump that can be correlated with an enhanced activity of the (Na+K)-ATPase. The possibility that this hypermetabolic state of the liver is related to the development of the pathological changes observed in experimental animals and in human alcoholics is suggested.

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Ethanol Metabolism and Enzyme Changes in Alcoholics with and without Hepatic Damage

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A great interest in ethanol metabolism related to metabolic alterations and toxic effects secondary to alcohol abuse has been observed in recent years (Hawkins and Kalant, 1972). Blood alcohol elimination rate has been shown to be significantly increased in chronically alcohol treated rats and in recently drinking alcoholics (Hawkins, Kalant and Khanna, 1966; Kater, Carulli and Iber, 1969; Ugarte, Iturriaga, Pereda and Pino, 1970). The increased blood alcohol clearance in recently drinking alcoholics disappears after two weeks of abstinence (Ugarte, Pereda, Pino and Iturriaga, 1972). However, in patients with steatosis without overt liver failure, a moderate but significant increase in blood alcohol removal was observed persisting after 15 days of abstinence (Ugarte *et al.* 1970; Ugarte and Valenzuela, 1971). Since all these patients drank more than 200 g of alcohol per day and no difference in the dietary survey could be recorded between alcoholics with steatosis and those with normal hepatic histology (Insunza, Iturriaga, Ugarte and Altschiller, 1971) this increase may be related to adaptive changes due to higher ethanol intake rather than dietary factors. Another possible explanation could be the presence of a pre-existent different ethanol metabolic rate in alcoholics developing liver damage. Racial differences in ethanol metabolic rate, which are probably genetically determined have been recently described (Fenna, Mix, Schaefer and Gilbert, 1971).

These findings have induced us to explore possible changes in enzymatic pathways involved in alcohol metabolism in both alcoholic patients and experimental animals. We have also studied the effect on ethanol metabolism of other drugs which may induce changes in enzymes related to alcohol oxidation.

Alcohol Dehydrogenase (ADH)

This enzyme, considered the most important in ethanol oxidation, was studied in liver biopsies of chronic alcoholics at pH 9.6 (Ugarte, Pino and Insunza, 1967) and also at pH 8.8 and 10.8 to differentiate between cases with normal and a typical ADH (von Papenberg and Aebi, 1965). The activity of this enzyme at pH 9.6 was significantly lower in alcoholics; however, no difference was found among patients with liver damage without liver failure and alcoholics with normal liver histology (Ugarte *et al.* 1967).

When the normal and atypical ADH was studied according to the von Wartburg pH screening test, a higher prevalence of the atypical enzyme was found in alcoholics with liver damage (Ugarte, Pino, Altschiller, Pereda, 1970). However the presence of the atypical enzyme did not correlate with ethanol metabolic velocity (Fig. 1). These results agree with those of several other recently reviewed reports both in man and in the rat showing no correlation between ADH activity and ethanol metabolic rate (Hawkins and Kalant, 1972). These studies demonstrate that variations in ADH activity do not explain the enhanced ethanol metabolism in chronic alcoholics. Other ethanol oxidizing pathways or mechanisms improving NAD regeneration, the rate limiting step in ADH activity, have been therefore investigated.

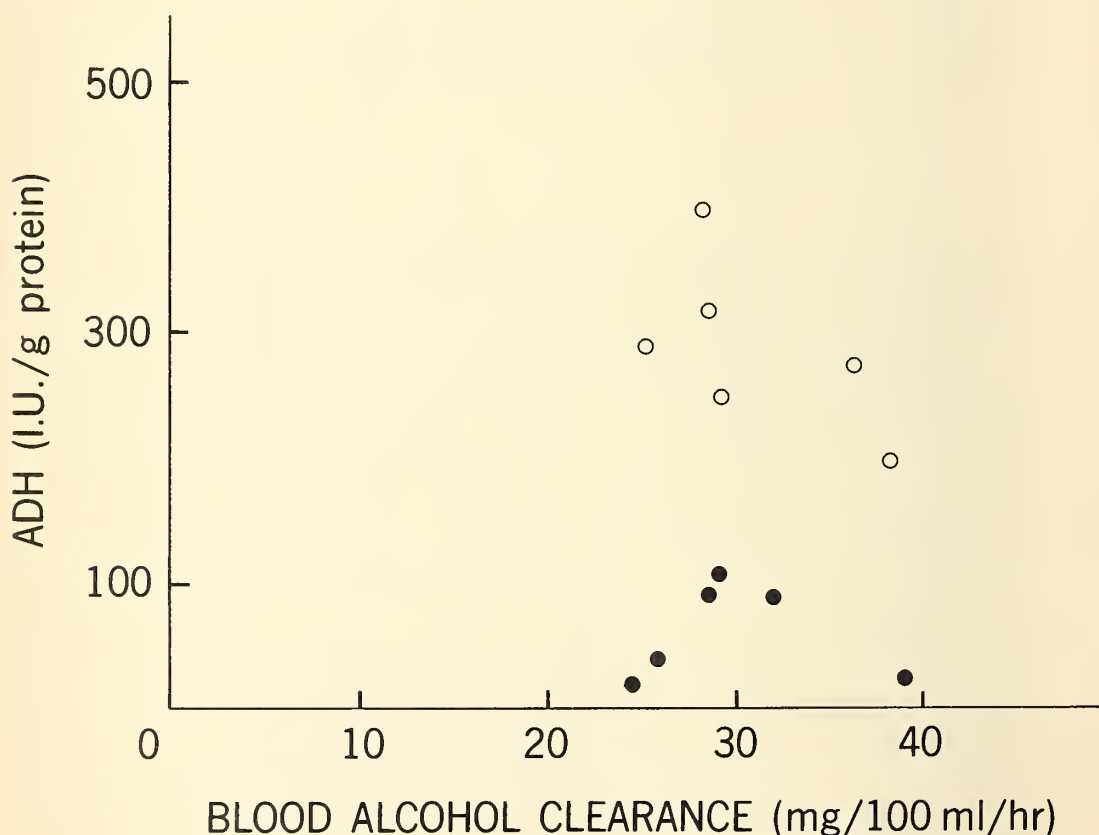


Figure 1. Lack of correlation between blood alcohol clearance and the activity of atypical or normal dehydrogenase in liver biopsies of recently drinking alcoholics. Each dot represents one case with the normal enzyme and each open circle a case with the atypical variant.

Hepatic Catalase

Hepatic catalase represents another ethanol oxidizing system not requiring NAD. The rate limiting factor in ethanol oxidation by catalase seems to be the presence of a H_2O_2 generating system (Lundquist, Svendsen and Petersen, 1963). Hydrogen peroxide may be formed from NADPH oxidation, a mechanism demonstrated by several investigators to explain microsomal ethanol oxidation (Roach, Rees and Creaven, 1969; Khanna, Kalant and Lin, 1970; Isselbacher and Carter, 1970; Thurman, Ley and Scholz, 1972; Lin, Kalant and Khanna, 1972). The increased number of peroxisomes observed in the liver of alcoholic subjects has suggested that ethanol oxidation — via catalase — may be operating in individuals exposed to prolonged excessive alcohol intake.

Normal or increased hepatic catalase activity has been found in chronic ethanol treated rats (von Wartburg and von Rothlisberger, 1961; Ugarte, Pereda and Itturriaga, 1969; Videla, Bernstein and Israel, 1973). Experiments in our laboratory have demonstrated that the increase of hepatic catalase activity in alcohol treated rats is dependent on the protein and alcohol content of the diet. Rats with high protein and high ethanol intake in the diet exhibit significant increase of hepatic catalase activity which correlates with an augmented ethanol metabolic rate (Table I).

TABLE I

EFFECT OF DIFFERENT ALCOHOL-DIET
COMBINATIONS ON LIVER CATALASE ACTIVITY

Alcohol-protein (.) combination	Increase in body weight (g) daily	Consumption of protein (%) of daily caloric intake)	Daily caloric intake	Alcohol intake (g/Kg BW)	Hepatic catalase (U/g wet liver)		
					controls	Exp.	p
High protein high alcohol (9)	0.81	18	37	24	92	166	.001
Low protein high alcohol (8)	0.31	12	34	23	56	75	.05
High protein low alcohol (13)	1.36	18.5	30	15	70	88	.10

(.) Each group compared with a pair fed control group. Alcohol was isocalorically replaced by sucrose. Alcohol was given in drinking water during 25 days.

However, we found that acetylsalicylic acid administration to rats which has been reported to increase the number of peroxisomes (Hruban, Swift and Slesers, 1966) also increases hepatic catalase but with no effect on ethanol metabolic rate (Table II). Hepatic catalase activity was also studied in liver biopsies of recently drinking alcoholics and in abstinent alcoholics (3 days of abstinence). There was no difference in catalase activity between recently drinking alcoholics and abstinent alcoholics and both groups were also

TABLE II

HEPATIC CATALASE ACTIVITY
AND ETHANOL METABOLIC RATE IN RATS
PRETREATED WITH ALCOHOL OR ACETYLSALICYLIC ACID

	Catalase U/g liver wet weight	p	E.M.R. mg/kg/h	p
Controls (9)	92 ± 5		198 ± 29	
Ethanol (10)	166 ± 14	<.001	314 ± 40	<.05
Controls (11)	60 ± 8		160 ± 20	
Acetyl salicylic acid (10)	108 ± 9	<.005	161 ± 19	NS

Mean ± Standard error; NS non significant.

TABLE III

HEPATIC CATALASE ACTIVITY IN ALCOHOL ADDICTS

	Kat.f. Units mg protein	S.E.
Control (surgical biopsy) (10)	.35	.04
Recently drinking alcoholics (8)	.31	.02
Abstinent alcoholics (18)	.36	.01

Differences not significant. Ugarte *et al* 1970.

not significantly different from controls (Table III). In these cases the protein content of the diet was unknown but probably deficient. These facts suggest that the augmented ethanol oxidation observed in recently drinking alcoholics and in abstinent alcoholics with steatosis without liver failure is not directly related to the increase in hepatic catalase activity.

Catalase may play a role if H_2O_2 is available in peroxisomes from lactate oxidation or NADPH oxidation. These conditions could conceivably exist in alcohol treated rats and not in rats pretreated with acetylsalicylic acid. The partial inhibition of ethanol oxidation in livers of alcohol treated by aminotriazole, a catalase inhibitor, seems to support this assumption (Tephly, Tinelli and Watkins 1969; Videla and Israel, 1970) although the specificity of this inhibitor is questionable (Lieber and De Carli 1970).

Role of Microsomal Enzymes and Mitochondrial Oxidation

Ethanol may be oxidized to acetaldehyde by the microsomal fraction of liver cells in the presence of NADPH and oxygen. The process is partially inhibited by carbon monoxide sharing similar characteristics with microsomal mixed-function oxidases (Orme-Johnson and Ziegler, 1965; Lieber and De Carli, 1968). Lieber and DeCarli (1970) have further

studied this microsomal system (MEOS) *in vitro* and *in vivo*, describing its differences from catalase and ADH and considering it specific for alcohol oxidation. Its role has been estimated at 20% of normal ethanol metabolism and up to 60% of the increased blood ethanol oxidation in chronic ethanol treated rats (Lieber 1973). However, Khanna, Kalant and Lin (1972) found in chronic ethanol treated rats that a high protein content in the diet maintained an increased ethanol metabolic rate while MEOS activity *in vitro* did not change. Moreover, it has also been recently demonstrated that microsomal ethanol oxidation is limited by the rate of H_2O_2 formation. The ethanol metabolic rate being higher in the presence of H_2O_2 generating system than in the presence of NADPH generating system (Thurman *et al.*, 1972; Lin *et al.*, 1972). These observations suggest that microsomal ethanol oxidation share some properties with catalase mediated alcohol oxidation or that as it has been suggested, microsomal preparations are contaminated with catalase (Roach *et al.*, 1969; Khanna *et al.*, 1970; Isselbacher and Carter, 1970).

While the identity and importance of the MEOS system is debated, Videla and Israel (1970) showed that uncoupling agents significantly increase ethanol metabolism in liver slices of normal rats. This fact indicates that mitochondrial NADH reoxidation is probably the rate limiting step in ethanol oxidation in the normal liver. Mitochondrial uncouplers, however, were ineffective in augmenting alcohol metabolism in liver slices of chronically alcohol treated rats. These results suggested that chronic ethanol treatment increased the oxidative properties of rat mitochondria rendering them insensitive to uncouplers.

Data heretofore discussed indicate that the increase in alcohol metabolism observed in chronic alcohol abusers is probably conditioned either by an increase in mitochondrial NADH reoxidation or by the induction of MEOS. In order to further assess the role of these mechanisms in augmenting alcohol metabolism we have studied the effect of chronic halothane inhalation in rats and meprobamate treatment in humans. Meprobamate, a microsomal inducer, did not modify blood alcohol clearance in humans (Table IV). Similar results have been reported with other drugs which induce MEOS activity (Khanna and Kalant, 1970; Khanna *et al.*, 1972). Both acute and chronic halothane administration to rats have been reported as impairing drug metabolizing enzymes, cytochrome P 450, NADP cytochrome C reductase and NADPH oxidase (Kunz, Schaudé, Schimassek, Schmid and Siesse, 1966; Davis, Schroeder, Gram, Reagan and Gillette, 1971). Chronic halothane inhalation in rats on the other hand enhances malic enzyme

TABLE IV

BLOOD ALCOHOL ELIMINATION RATE
IN ABSTINENT ALCOHOLICS AFTER
15 DAYS OF MEPROBAMATE TREATMENT

	Rate of ethanol elimination (β) mg/100 ml/h
Abstinent alcoholics (23)	19 ± 1.7
Meprobamate treated abstinent alcoholics (10)	17 ± 2.0

Subjects studied after 15 days of abstinence and 10 days of Meprobamate (1.2 g per day) treatment.
Alcohol 1.0 g per Kg BW infused intravenously.

activity and also the activity of several mitochondrial oxidative enzymes specially α -glycerophosphate dehydrogenase (Kunz *et al.* 1966).

We studied blood ethanol removal in rats exposed for 15 days to inhalation of 0.5% halothane (1 hr daily under standardized conditions) with the aid of a fluotech machine (Ugarte, Pino, Pereda, Iturriaga, 1973). A significant increase of approximately 30% in blood ethanol clearance was observed in halothane treated rats (Table V). The activity of the malic enzyme in the liver and adipose tissue and of the hepatic mitochondrial α -glycerophosphate dehydrogenase were enhanced in rats exposed to halothane, whereas no significant changes in liver ADH and Na + K stimulated adenosine triphosphatase activities were observed (Table VI).

TABLE V

RATE OF BLOOD ETHANOL ELIMINATION
IN CHRONIC HALOTHANE TREATED RATS

Ethanol elimination rats	Normal rats (9) mean \pm SE	Halothane treated rats (7) mean \pm
mg/100 ml/h	24.4 \pm 2.3	33.1 \pm 4.5*
mg/Kg/h	292.1 \pm 19	378 \pm 13**

* $p < 0.05$; ** $p < 0.01$

Ugarate et al. 1973

TABLE VI

MALIC ENZYME, MITOCHONDRIAL ALPHA
GLYCEROPHOSPHATE DEHYDROGENASE,
ADH AND NA + K ATPASE ACTIVITIES
IN THE LIVER OF CHRONIC HALOTHANE

	Normal rats	Halothane treated rats
Malic enzyme, liver IU x mg/protein x 10^2	1.03 \pm 0.23 (8)	6.26 \pm 1.26* (9)
Malic enzyme, adipose tissue IU x mg/protein x 10	0.75 \pm 0.33 (9)	1.84 \pm 0.71** (8)
alpha-Glycerophosphate dehydrogenase IU x mg/protein x 10^2	1.48 \pm 0.27 (9)	2.11 \pm 0.27** (9)
ADH IU x mg/protein x 10^2	4.18 \pm 0.32 (9)	3.63 \pm 0.42 NS
Na + K ATPase mg P/mg protein	0.61 \pm 0.21 (6)	0.57 \pm 0.01 NS

Values shown are mean \pm S.E.M.

* $p < 0.0025$; ** $p < 0.05$; NS not significant

The augmented ethanol metabolism seen in chronic halothane treated rats seems then highly unlikely to be mediated by MEOS considering that NADPH oxidase activity and cytochrome P 450 are diminished in this condition (Kunz *et al.*, 1966; Davis *et al.*, 1971). The increased blood alcohol removal observed in chronic halothane treated rats may be related to enhanced mitochondrial NADH reoxidation. A significant increase in mitochondrial α -glycerophosphate dehydrogenase was observed. Other substances exhibiting a similar effect are clofibrate and ethanol itself (Kähänen, Ylikari and Hassinen 1971; Israel, Videla, MacDonald and Bernstein, 1973). These substances have been shown to increase mitochondrial oxidations and blood ethanol clearance. Data reported recently by Videla *et al.* (1973) show that chronic ethanol administration in rats results in a decrease in mitochondrial phosphorylation potential and a concomitant increase in liver tissue respiration. This extra respiration could be blocked by ouabain — an inhibitor of Na + K stimulated ATPase (Bernstein, Videla, Israel, 1973). The activity of this enzyme was found elevated in both chronic alcohol treated and hyperthyroid rat livers (Israel *et al.*, 1973). Based on these findings and on the observation that acute administration of alcohol increase 131-thyroxine uptake by the liver, it has been suggested that chronic alcohol administration may enhance mitochondrial oxidations establishing a functional hyperthyroid state in the liver. Although we have found blood ethanol removal greatly augmented in hyperthyroid nonalcoholic patients, we have not found increased Na + K stimulated ATPase activity in rats chronically treated with halothane (Table VII). However, the increase in ethanol metabolism attributed to enhanced mitochondrial oxidative capacity mediated by an augmented Na + K stimulated ATPase is much greater than that which we observed in chronic halothane treated rats.

TABLE VII

ETHANOL METABOLIC RATE IN HYPERTHYROID PATIENTS

Case	B.M.R. %	P.B.I. $\mu\text{g}/100\text{ ml}$	E.M.R. $\text{mg}/\text{kg}/\text{h}$
1	+80	15	380
2	+120	18	360
3	+70	12	275

B.M.R.: Basal metabolic rate; P.B.I. Protein bound iodine.
E.M.R.: Ethanol metabolic rate (Normal $162 \pm \text{mg}/\text{kg}/\text{h}$).

Another possible mechanism influencing ethanol metabolic rate through changes in the cytosol redox state of liver cells could be related to the enhanced malic enzyme activity. Reoxidation of NADH may result from transhydrogenation between NADH and NADPH when malic enzyme and lactic dehydrogenase share pyruvate as a common reactant (Veech, Eggleston and Krebs, 1969). Similarly another transhydrogenation reaction requiring ATP and mediated by pyruvate carboxylase, malic dehydrogenase and malic enzyme may regenerate NAD yielding NADPH (Wise and Ball 1964). These transhydrogenation reactions may explain that at the same time that NADH is reoxidized enhancing ethanol metabolism NADPH is provided for lipogenesis. Chronic halothane treated rats have been shown to develop hepatic steatosis (Kunz *et al.*, 1966), a finding confirmed in our study.

These reactions probably do not explain completely an increase of 100% in ethanol metabolism as seen after chronic ethanol intake. However the moderately augmented blood ethanol clearance in abstinent alcoholics with greater susceptibility to hepatic steatosis could be secondary to this transhydrogenation mechanism. The increase in blood alcohol clearance in humans after fructose administration has also been attributed to this pathway (Thieden, Grunnet, Damgaard and Sestoft, 1973). In support of this hypothesis is also the finding of an increasing NADPH/NADP ratio after chronic ethanol treatment (Kalant, Khanna and Loth 1970).

Malic enzyme activity has been found elevated in adipose tissue of chronic halothane treated rats (Ugarte *et al.*, 1973) and of chronic alcohol treated rats (Solodkowska, Alvarado and Mardones, 1972). This finding, too, may be related to increased ethanol metabolism and lipogenesis in adipose tissue. In this respect enhanced alcohol metabolism and ethanol incorporation in adipose tissue in rats chronically treated with ethanol or carbon tetrachloride have been reported (Campos, Solodowska, Muñoz, Segovia, Riquelme, Cambrano and Mardones, 1964; Solodowska, Alvarado, Muñoz, Jara, Segovia and Mardones, 1966).

SUMMARY

Ethanol metabolic rate is significantly enhanced in recently drinking alcoholics. A moderate but significant increase in blood alcohol removal has been observed in abstinent alcoholics with steatosis without liver failure compared with alcoholics with normal liver histology. These differences in alcohol metabolic rate do not seem related to ADH activity or catalase activity. They could be explained either by the operation of an induced microsomal enzymatic pathway or by increased NADH regeneration. The lack of effect of meprobamate on blood alcohol clearance in humans and the increase in blood ethanol in rats chronically treated with halothane, a microsomal inhibitor, suggest that a microsomal enzyme system is not very important in augmented alcohol metabolism. The enhanced mitochondrial α -glycerophosphate dehydrogenase in rats with increased ethanol metabolism after treatment with halothane and other drugs gives support to work reported by others suggesting that enhanced mitochondrial oxidations influencing NAD regeneration may be the main mechanism augmenting alcohol metabolism. The increase in malic activity in liver and adipose tissue of subjects with augmented ethanol metabolism suggests that transhydrogenation reactions between NADH and NADPH may be active in NAD regeneration. This mechanism may favor hepatic steatosis in alcoholics while moderately increasing blood alcohol clearance.

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Free Liver Cells in the Study of Albumin Synthesis and the Effect of Ethanol

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INTRODUCTION

Berry and Friend (1969) devised a method for the isolation of hepatocytes. This method has been widely applied to obtain cells for short term metabolic studies and for long term hepatocyte cultures. Attempts to use these cells to synthesize albumin at a linear rate comparable to the isolated perfused liver hitherto have given disappointing results (Weigand, Muller, Urban and Schreiber, 1971; East, Louis and Hoffenberg, 1973). Synthesis was linear only up to 4 hours and by that time most of the cells had altered membrane permeability as shown by a large number of cells staining with trypan blue. Furthermore, with maximal supplementation synthesis was only 10-20% of the *in vivo* state. Also, there was no detail concerning the content of adenosine triphosphate (ATP) and ribonucleic acid (RNA), and the synthesis of urea, the last of which would reflect the rate of gluconeogenesis.

Ethanol has been shown recently to reduce plasma protein synthesis in the isolated perfused liver (Rothschild, Oratz, Mongelli and Schreiber, 1971) and *in vivo* (Jeejeebhoy, Phillips, Bruce-Robertson, Ho and Sodtke, 1972). However, no studies have been done to show that it would depress albumin synthesis directly in isolated hepatocytes.

In this paper we describe an isolated rat hepatocyte system which synthesizes albumin at a linear rate up to 6 hours (and in amounts to at least 2.5 times the values observed with most preparations of isolated perfused liver) and more recently at a linear rate up to 12 hours (and in amounts up to 4 times most values observed in the isolated perfused liver). Albumin synthesis has been depressed by the addition of ethanol at levels which may be present in the portal blood of man when he is intoxicated. (Dr. Ivan T. Beck, personal communication.)

MATERIALS AND METHODS

Preparation of Isolated Hepatocytes

A modification of the method of Berry and Friend (1969) was carried out using livers from 148-195 g Wistar rats.

Technique of Culture

In the first series of experiments (series I), the cells were suspended in Ham's F10 nutrient mixture (Ham, 1963) to which was added 17.5% heat-inactivated horse serum. The concentration of cells varied from $1.2\text{--}2.2 \times 10^6$ cells/ml of medium. The cells were divided into equal aliquots which were placed in two separate 250 ml plastic Erlenmeyer flasks, one served as a control and the other was treated with ethanol. In this way each set of ethanol-treated cells was compared to its own control. The flasks were shaken in a water bath while being constantly gassed with 95% O_2 + 5% CO_2 . The pH was adjusted to 7.4 by adding 0.1N $NaHCO_3$ at 2 hourly intervals. This was required especially in the ethanol-containing flask. The pO_2 in series I was about 340 mm Hg. A second series (series II) was conducted in 250 ml glass containers constantly stirred with a magnetic stirrer and gassed with an equal mixture of 95% O_2 + 5% CO_2 and 95% N_2 + 5% CO_2 to give a pO_2 of 130-150 mm Hg in the medium. This series comprised two ethanol experiments and five further control experiments. The latter were done in order to compare the results of cell suspensions divided in the same way as in the ethanol experiments, but incubated in pairs without differing treatments in order to ascertain the variability within controls. Three experiments were done with Ham's F10 nutrient mixture and two with Waymouth's medium MB 752/1 (Waymouth, 1959). (Both media were obtained from Grand Island Biological Co., Grand Island, New York). To each flask 100 μ c of 3H -L-Valine-GL (specific radioactivity 6.8 c/nmole; International Chemical & Nuclear Corp., Irvine, Cal.) was added. Measurement of urea was done by a microConway method (Obrink, 1955). Albumin was measured by solid phase radioimmunoassay (Askenase and Leonard, 1970). Incorporation of 3H -Valine into total hepatocyte protein was determined by the method of Mans and Novelli (1960). RNA and DNA were determined with the method of Maggio, Siekevitz and Palade (1963). ATP levels were determined in control suspension by Lowry's method (Lowry and Passoneau, 1972) using a Turner fluorometer.

RESULTS

Percentage of cells stainable with trypan blue. — In series I controls, 73% of the cells present, on the average, were unstained after 6 hours of incubation and 46% after 12 hours of incubation. There was no significant difference ($P > 0.1$) between control and ethanol-treated cells.

In series II, the percentage of control cells unstained after 12 hours of incubation was 81% on the average.

Albumin synthesis. — In controls incubated in Ham's F10 medium the mean albumin synthesis was 28.4 mg/h/100 g hepatocytes which is equivalent to about 3.5 mg/h/300 g rat. In those controls incubated in Waymouth's medium the synthesis was

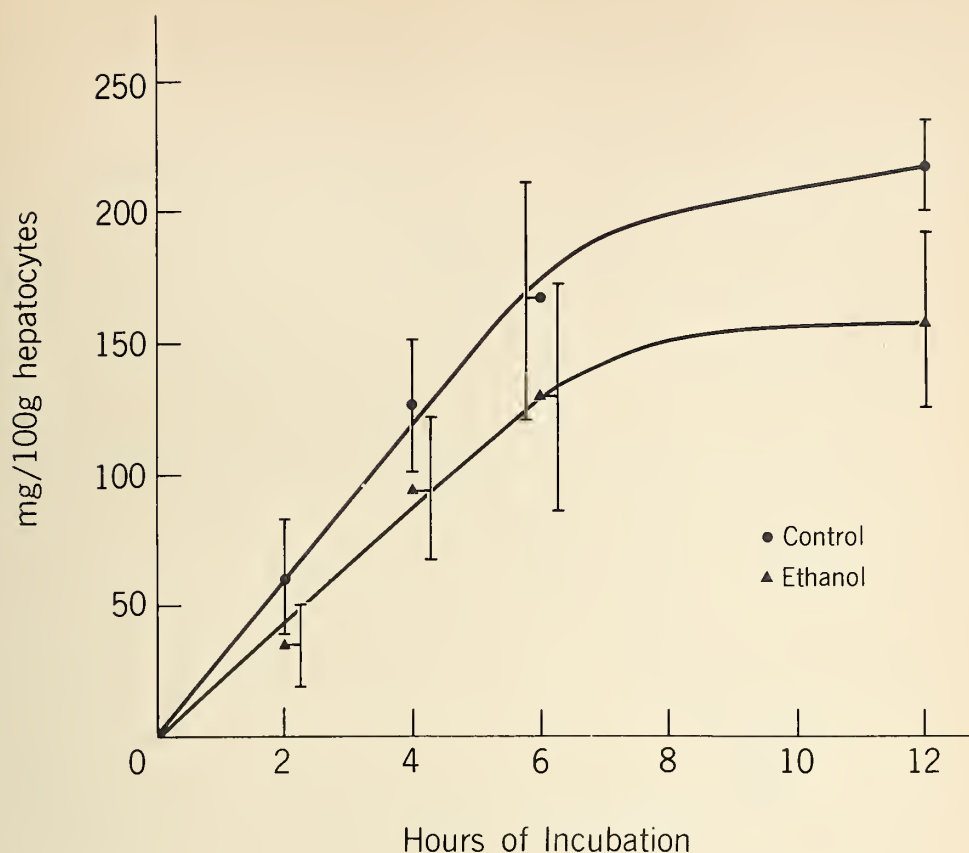


Figure 1 Albumin synthesis by isolated hepatocytes in the presence of ethanol (500-1000 mg% at 0h, decreasing to 130-770 mg% at 6h and to still less at 12h). The abscissa is length of incubation in hours. The ordinate is synthesis of albumin as mg per 100 g of hepatocytes. Values are given as the means and standard deviations of 6 experiments.

46.6 mg/h/100 g hepatocytes when computed from 0-6 hours and 43.8 mg/h/100 g hepatocytes when computed from 0-12 hours. Hence synthesis in series II with Waymouth was essentially linear for the first 12 hours.

The results of ethanol treatment are shown in Figure 1. There is a progressively increasing depression of synthesis with time, up to twelve hours of incubation, when the ethanol-treated group showed 28% less synthesis than the control group. The pair difference was statistically significant ($0.02 > P > 0.01$).

Incorporation of ^3H -Valine into total hepatocyte protein. — The results are depicted in Fig. 2. Again the ethanol-treated group showed a progressive depression of uptake compared to the control group. The ethanol-treated group was 26% lower than the control one after 12 hours of incubation. This pair difference was statistically significant ($P < 0.01$).

Urea synthesis. — In controls urea synthesis was 15.4 ± 4.67 (mean \pm S.D.) and 11.9 ± 2.38 $\mu\text{moles/h/g}$ hepatocytes in ethanol-treated cells. The difference was not statistically significant ($P > 0.1$).

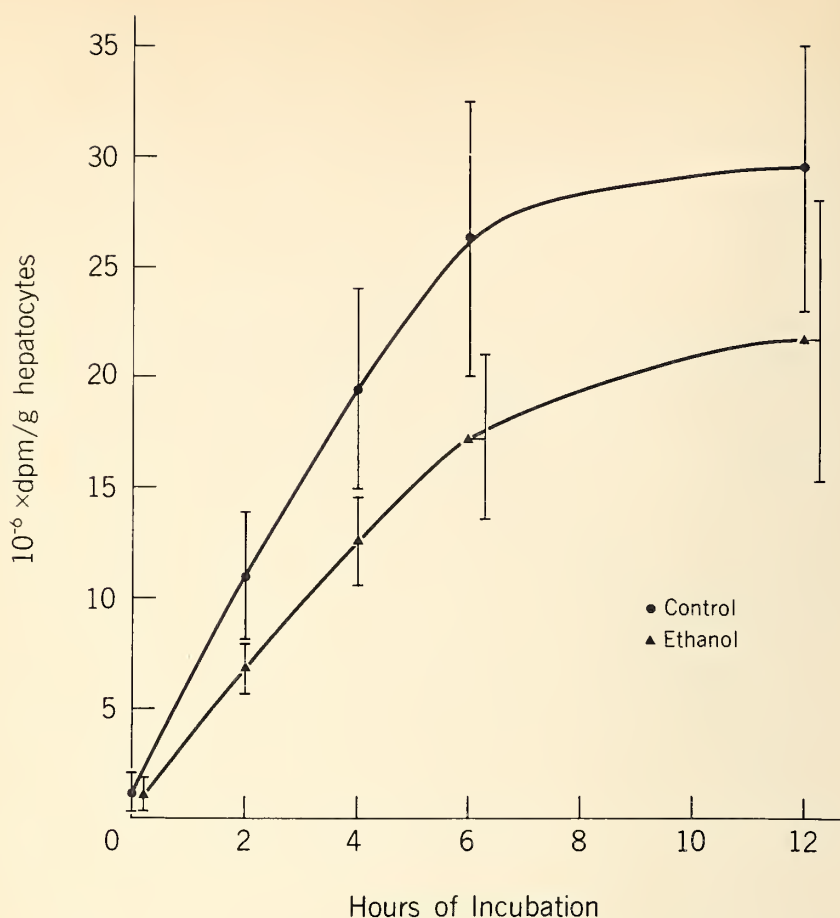


Figure 2 Incorporation of ^3H -Valine into total hepatocyte protein. The abscissa is length of incubation in hours. The ordinate is $10^{-6} \times \text{dpm/g hepatocytes}$. Values are given as the means and standard deviations of 6 experiments.

ATP levels. — These were measured only in controls and the value was $3.25 \mu\text{moles/g}$ hepatocytes after 6 hours' incubation and $2.52 \mu\text{moles/g}$ after 12 hours' incubation.

RNA/DNA ratios. — This ratio in controls was 3.11 after 24 hours of incubation.

Electron microscopy. — The cells showed excellent preservation of ultrastructure after 12 hours of incubation with intact surface microvilli, endoplasmic reticulum, mitochondria and nucleus in the controls.

DISCUSSION

These results with ethanol are truly indicative since they have been obtained in a system that we feel is valid since the technique defined above results in metabolically active hepatocytes with ATP levels and RNA/DNA ratios comparable to those in the *in vivo* state (Bucher *et al.*, 1964, Bloxam, 1971). The ultrastructure after 12 hours' incubation was also excellent and in series II over 80% of the cells at this time had membranes

impermeable to trypan blue. The albumin synthesis in this isolated hepatocyte system using Ham's F10 medium (3.4 mg/h/300 g rat) equals the best results in the isolated perfused liver as reported by John and Miller (1969) and Hoffenberg, Gordon and Black (1971) although less than the recent value of 6.3 (Tavill, East, Black, Nadkarni and Hoffenberg, 1973). When compared in terms of liver weight, albumin synthesis is 2.2 times that reported for the perfused liver by Oratz, Rothschild, Burks, Mongelli and Schreiber (1973). With Waymouth's medium, albumin synthesis is even better, being 46.6 mg/h/100 g hepatocytes or equivalent to 5.6 mg/h/300 g rat, which is closer to Tavill's value just mentioned (in the perfused liver) and about half the *in vivo* value (Jeejeebhoy *et al.*, 1972). The urea synthesis of 15.4 μ mol/h/g liver is higher than that observed in the perfused liver where values about 10.6 μ mol/h/g liver have been reported (Bloxam, 1971).

Against this background of excellent viability the addition of ethanol, to give a mean concentration between 250-500 mg%, resulted in a significant depression of albumin synthesis and of the incorporation of ^3H -Valine into total liver protein, but not a statistically significant reduction in urea production, thus showing a direct effect on the liver without definite nitrogen sparing action on amino acid catabolism. These changes were not associated with gross alteration in membrane permeability.

The reduction in synthesis was not due to a lack of release of protein from hepatocytes as suggested by Isselbacher and Greenberger (1964) because the reduction in albumin synthesis and incorporation of ^3H -Valine was noted in homogenized suspensions where intracellular protein was included.

Clearly the hepatocyte suspension technique mentioned above is capable of providing a model viable for 12 hours. This period of time is of longer duration than those reported for the perfused liver model (except for that of John and Miller, 1969). Our suspension is capable of albumin and urea synthesis comparable to the best perfused liver models. The ATP levels and RNA/DNA ratio are comparable to *in vivo* values and structural viability is excellent. Our preparation appears to be an improvement upon those isolated hepatocyte systems which are capable of synthesizing albumin and which have been previously published by Tavill *et al.* (1973) and East *et al.* (1973).

Using this model a direct toxic effect of ethanol on hepatocytes can be demonstrated at levels which may be found in the portal blood of man.

SUMMARY

Preliminary results for the effect of ethanol on the synthesis of albumin and urea in a rat hepatocyte suspension are presented. The best control results to date have been obtained with Waymouth's MB 725/1 medium and have demonstrated that these liver cells produced albumin for 12 hours at a rate close to that of the best reported for the isolated perfused liver and about half of the *in vivo* rate. Urea metabolism was more active than has been reported for the isolated perfused liver. Exclusion of trypan blue, RNA/DNA ratios, ATP levels and electron microscopy results suggested satisfactory viability and biochemical function at 12 hours and retention of structural features for 24 hours.

Addition of ethanol at the high concentration of 250-500 mg% to the medium showed a significant depression of albumin synthesis without a significant effect on urea synthesis and membrane permeability to trypan blue. This suggests to us that there is a direct toxic effect of ethanol upon the hepatocyte.

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Concluding Remarks

H. Kalant

The function of concluding remarks at a conference of this kind is perhaps a matter of some uncertainty. We have had our heads so filled with thoughts, facts and discussions during the last few days that to try to recapitulate them here would serve no useful purpose. A detailed critique of all the papers would be at best presumptuous, and at worst both presumptuous and foolhardy. What I propose to say now, therefore, really purports to be nothing more than a personal impression of, and speculations on, some of the main points that have come out of the meeting.

Research in any field has its *zeitgeist*. I use this term purposely, because in the area of alcohol research it might be ill advised to say "spirit of the times." This *zeitgeist* is recognizable only in retrospect. In the 1950s, there was a heavy emphasis on the role of nutritional disturbances in the damage produced by alcohol. The work of Hartroft, Klat-skin, Patek and Post, and many others, contributed important concepts which have either become accepted parts of our knowledge, or continue to be subjects of active research.

In the 1960s, however, the major emphasis shifted, to a large extent due to the very important work of Lieber and his colleagues, Isselbacher, Freinkel and others. Though the importance of nutritional disturbances was accepted, there was progressively greater interest in the consequences of disturbance of intermediary metabolism in the liver due to the metabolism of alcohol itself. There was a very rapid and extensive clarification of the impact of alcohol metabolism, by whatever the enzymatic routes may be, on the metabolism of other substances, and of the relation of the metabolic disturbance to the production of hepatic damage. Rather naturally, the greatest emphasis was placed on the type of hepatic damage which was most demonstrably connected in the shortest possible times with the disturbances in metabolism. This meant an emphasis, for obvious reasons, on fatty liver.

During the past few days, in the sessions of this symposium, I have sensed that the emphasis is again changing, and that perhaps research during the 1970s will be directed primarily toward the role of alcohol itself as a hepatotoxic substance, as distinct from its role as a metabolic substrate which disturbs the normal flow of other metabolic processes. The clinical and epidemiological observations which provided the rationale for this new direction of research were very well outlined by Dr. Lelbach in the first session. His paper stressed the importance of total alcohol exposure — in other words, intensity of exposure multiplied by its duration — in the production of gross, apparently irreversible structural damage in the liver. This was supported in the epidemiological papers by Dr. Schmidt and Dr. Rankin. Though they took account of the arguments concerning the differences among individual alcoholic beverages, and factors such as sex and genetic makeup, they were led inevitably back to the importance of alcohol itself. The blood alcohol levels achieved, and the time over which they are maintained, seem to be very important and possibly critical determinants in relation to the production of the more serious types of clinically recognized liver disease.

This was borne out by several of the other papers. Dr. Galambos, for example, in his follow-up study of patients with hepatic damage diagnosed by biopsy, found that the subsequent evolution of the disease correlated primarily with whether or not they continued to drink, rather than with the stage of disease at the time of entrance, with the dietary or other treatments, or with other variables that he considered. Dr. Lieber, in presenting the beautiful studies on baboons, demonstrated convincingly full-blown hepatic damage in animals which had taken over 50% of their calories as alcohol. These animals obviously had a greater total alcohol exposure than those in the earlier studies in which the caloric contribution of alcohol, and hence the total alcohol exposure, were set at lower levels.

Despite differences in semantic interpretation of nutritional vs. direct alcohol hepatotoxic mechanisms, Dr. Hartroft also agreed on the importance of the total alcohol exposure. Drs. Takeuchi and Takada again stressed that recognizable structural alterations in the liver, as distinct from metabolic disturbances, could be produced in their experimental animals only if periods of high blood alcohol level, and therefore high exposure of the liver cell to alcohol, were superimposed on sustained nutritional or metabolic disturbance related to alcohol. There seemed, to me at least, to be a consensus that we are dealing now with a period in which increasing emphasis in research will be placed upon the significance of the alcohol level, or the level of alcohol exposure of the liver cell, in the production of damage.

This approach necessarily introduces changes in certain other broader concepts. One needs to re-think experimental models and pathogenic concepts in rather more ecumenical terms that we have in the past. If we talk about higher blood alcohol levels, we must consider not only direct effects of alcohol upon the liver cell, but also a whole series of systemic physiological changes related to intoxication, which modify the impact of alcohol and of alcohol metabolism, and probably also of nutritional disturbance, on the liver cell. The effects of alcohol, in other words, can not be considered in relation to the liver cell alone. We must begin once more to think of the liver as an organized structure with blood flow, acted on by hormonal and other influences arising in the rest of the body. One needs to think of the secondary, or tertiary, or even more remote effects of a variety of physiological processes related to sustained high blood alcohol levels, which may act on the liver cell. Dr. Marleau showed, for example, very dramatic end pictures of vascular disturbance, and their importance as determinants of death or amenability to treatment in patients with established liver disease. But his work also

emphasized the need for a fine analysis of intimate blood flow, in other words blood flow measured not only in terms of proportional flow through portal vessels and hepatic artery, but also of the rate of blood flow within the sinusoids and exposure of the liver cells to blood of varying compositions. This tied in very neatly with Dr. Rappaport's extremely informative review of the basic vascular physiology of the liver, the importance of oxygen gradients, pressure gradients, and the functions of liver cells in different zones.

Other papers, in other sessions of this symposium, contributed very importantly to emphasizing the same point: that the effect of alcohol on the physiology of the liver cell can not be understood in isolation. We can not understand alcoholic liver disease exclusively in terms of the direct effect of alcohol *per se*, but only as part of the complex of physiological disturbances in the whole body. For example, Dr. Jeejeebhoy's paper on the effects of alcohol on the synthesis of serum albumin by isolated liver cells *in vitro*, and the effect of oxygen pressure on it, provided strong (even if indirect) supporting evidence for the concepts which Dr. Rappaport had advanced. The changes in pO_2 which he used *in vitro* have their parallel in changes of blood flow *in vivo*. This emphasizes again the point of Dr. Marleau's presentation, that our understanding of the functional damage in the cirrhotic patient will not be significantly advanced until we are able to measure the amount, composition and velocity of blood flowing through individual sinusoids in different parts of the liver. This, it seems to me, may well become one of the greatest challenges in this important area of research. Dr. Hartroft raised the possibility that the cirrhotic nodule may be, in fact, an overgrown acinus. He wondered whether the nodule is simply a regenerating acinus which has somehow been freed of the physiological controls which limit the growth and proliferation of normal liver cells to a point determined by the regional changes in blood composition. Here again, I think this stresses the need for studies of the factors which determine vascularization of the liver, and vascularization of regenerating nodules, the functional significance of blood flow, and the very important question of developing methods suitable for this type of analysis.

Another topic which has been very prominent in the overall discussion is the nature of the disease process itself that we are studying. I think we all agree that our primary concern in terms of the ultimate well-being of the patient is the necrotic and inflammatory lesion, which carries as its end result a disturbance of liver architecture and of liver function. Dr. Farber, in his review of general processes of cell damage, did us all an extremely important service in stressing that some of the process is probably not a non-specific phenomenon, not just a running down of the cell in general, but probably a fairly specific reaction or group of reactions related to imbalance between certain critical processes going on within the cell. He raised the possibility, for example, of overproduction of some protein or proteins relative to their rate of utilization. A prime candidate for our attention in this respect might be the protein constituents of cell membranes, both plasma and intracellular membranes, which affect cell integrity and cell enzymatic activity, and are critically important in the normal maintenance of the cell equilibrium.

Here again, echoes of this theme came up in several sessions during the meeting. Dr. Plaa offered a very ingenious suggestion for attempting to define the imbalance by isolating the various steps in it through some process such as hypothermia, or some other means of slowing down the whole process leading to cell degeneration and cell death. But even this approach, while it may help, will require a considerably clearer definition of the problem before it can be utilized. For example, Drs. Rubin, Ugarte and Israel all raised the question: which processes should we be looking at? What changes in a diseased liver constitute adaptive changes which protect the cell against damage, and which changes are

actually contributory to pathogenesis? For example, Dr. Ugarte and others emphasized during the meeting that an increased rate of alcohol metabolism is seen in the alcoholic patient, as well as in the animal treated chronically with alcohol; but is this a protective process or does it in fact contribute to the production of pathology? The problem here, I think, is that until one can define more sharply the actual steps immediately preceding cell death, one has to work backwards, and one doesn't really know by looking at the early changes which are protective and which are damaging. I think this points out the need to identify even more specifically, in the terms which Dr. Farber emphasized, what really leads to cell death.

Dr. French's very elegant presentation on the composition and formation of hyaline bodies also raises the possibility that these bodies represent an accumulation of normal component which for some reason is not being utilized in the normal fashion, and hence accumulates and gives rise to abnormal agglomeration within the cell. Possibly this is an instance of the sort of accumulation of protein that Dr. Farber spoke of, through production in excess of its utilization. One might consider the possibility, or the speculation, that we are dealing with material which, as Dr. French suggested, normally attaches to mitochondria or other organelles and regulates their distribution and localization within the cell, their relationship to each other and hence the relationship of the various metabolic processes which they carry out. Does this buildup of hyalin reflect a change in the membranes of the organelles and the plasma membrane itself, such that the fibrillar material can not attach to them in the normal way? Are we seeing primarily a change in the composition of the membrane rather than in the production of the fibrillar protein, such that there is impaired utilization of the materials and consequent abnormal accumulation, because they can not connect with their normal point of attachment? This is a question which can be raised at present only as a stimulus to new approaches, and a demonstration of the futility of concentrating on the accumulation of a material within the cell, in the absence of specific knowledge of what the normal function of that material may be.

Dr. Plaa described the effect of alcohols on toxicity due to carbon tetrachloride and other halogenated hydrocarbons. He pointed out that there was a selective effect on the microsomal systems involved in the activation of these hydrocarbons to produce the substances which are directly responsible for the damage. Dr. Lieber confirmed this. The question that Dr. Plaa raised was whether induction of metabolism alone was really enough to account for the effect, or whether there was also some direct pharmacological interaction between alcohol and some microsomal membrane-bound enzymes, to account for the greater enhancement of toxicity which was seen. Dr. Lieber's presentation echoed this theme in a sense, by emphasizing the prominent changes in microsomal membranes and membrane-bound enzymatic activities which are found in subjects treated chronically with alcohol. Once again, however, we are left in the dilemma of trying to decide which of the changes are adaptive and which of the changes are in fact pathogenetic.

The same question is raised in the presentations by Drs. Rubin and Israel on the alterations in mitochondria, which partly represent metabolic adaptation and partly structural and functional impairment. Moreover, some of the changes found may represent alterations in the membrane functions of these organelles themselves, and others may reflect changes in the metabolic control and regulation of the processes which are carried on in these structures. A good example is provided in Dr. Israel's study of the alteration of phosphorylation potential as a determinant of increased mitochondrial oxidative activity in the livers of animals treated chronically with ethanol. The change in phosphorylation potential appears to be caused by increased Na⁺K-ATPase activity in the cell

membrane. One could say that the change in enzyme activity in the cell membrane is adaptive, and enables the cell to compensate for disturbances in intracellular cation concentration produced acutely by alcohol. Yet chronically the consequences of the increased ATPase activity may contribute to pathogenesis in terms of altering mitochondrial function. There may be an increased rate of oxidation of alcohol, and yet, at the same time, increased disturbance of other metabolic functions not yet explored, which might contribute to pathogenesis.

Another major area in this conference has been the question of what constitutes the stimulus to fibrosis in the liver. Given the fact that there are necrotic zones, and there are vascular disturbances, what leads to the third major component of the pathological picture we see? Is fibrosis again the end-result of a rate imbalance? Dr. Mezey talked about the possibility of faster synthesis of collagen in the liver during active alcoholic hepatitis. But, as Dr. DiLuzio suggested, there is another possible way of looking at the data. Is the relation between the striking increase in proline hydroxylase activity in the liver, and the relatively small increase in hydroxyproline excretion in the urine, a reflection of a dilution of the newly formed hydroxyproline in all the rest of the body? Or does it reflect a difference between rates of formation of hydroxyproline and its utilization in the process of fibrosis? Does the amount of collagen itself present at any given time give a falsely static picture that really represents a kinetic imbalance between formation and removal? Is the picture of fibrosis really due to a failure of removal rather than an excessive formation of these substances? In attempting to resolve this, one has to answer three important questions: what is the stimulus to collagen formation, what is the mechanism of its removal, and what determines the balance between them? We can even raise again, in this connection, a question which we asked earlier: is increased collagen synthesis a sign of repair, or a step in pathogenesis?

Dr. Mezey pointed out that increased collagen synthetic activity, or increased activity of an enzyme probably related to collagen synthesis, is most marked during the stage of active hepatitis. This view was echoed in the presentations by Drs. Rubin and Lieber, Leevy, and Galambos. The various phenomena which they described all indicated that changes which might be conducive to ultimate structural damage were most marked during the stage of active hepatitis. But this is also the time when other biochemical changes, such as those which give rise to the fatty liver, are most marked. Is the fatty liver itself, then, a stimulus to fibrosis, to necrosis, to inflammation and the rest of the sequence, or is it merely an incidental manifestation of the process which really triggers these? Dr. Lieber pointed out correctly that we are not yet in a position to draw firm conclusions, and that one has still to reserve judgment. Yet the evidence does seem to be accumulating that what we should be looking for is some event which correlates in time with the fatty liver, but which is perhaps not a direct consequence of the fat accumulation itself.

Dr. Galambos pointed out that we also can not look for the answer in a difference in the fibroblasts themselves. When these were removed from the liver and grown in culture, one could not identify any abnormality in fibroblasts from cirrhotic livers in comparison with those from non-cirrhotic livers that would explain the greater fibrosis and destruction of lobular architecture characteristic of alcoholic cirrhosis. Dr. Lieber raised the question of a metabolic stimulus such as lactate. Dr. Leevy presented his extremely interesting work on altered lymphocyte reactivity, and the possibility that this might reflect an abnormal response to hepatic antigens. Yet in the last analysis I think we must still recognize that the stimulus to fibrous tissue proliferation itself has not been identified. We don't even know, for example, the tissue source of the abnormal glucos-

aminoglycans of which Dr. Galambos spoke, or the antigen proposed by Dr. Leevy. Drs. Lieber, Takada, Rubin and Plaa all pointed out that we are dealing with membranes of various types — microsomal, mitochondrial, plasma membranes — possibly from cells other than the hepatocyte, possibly Kupffer cells or other cells in the liver.

We are dealing with a complex organ, of which each cellular element has a variety of constituents that might provide the stimulus to fibroblast proliferation, and so far we have no way of knowing which component is responsible. The chemical characteristics of the disease process itself, the alterations in glucosaminoglycans or other constituents of fibrous tissue isolated from the liver or produced by liver cells in culture, seem to be, I think, a reflection rather than an explanation of the process of fibrosis. We are dealing with a variety of signs which reflect an increased fibrogenic activity. While they may be useful empirically in identifying the onset of the process, and also in understanding its essential steps, I think it would be rash to say that any of them has yet indicated to us the causal or even the fundamental pathogenetic steps in the generation of the fibrous response.

One must even consider whether characteristics other than those which have so far been looked at are of primary importance. We may have to come back to vascular factors such as pO_2 or blood flow, because, as Dr. Galambos pointed out, it may even be that the hepatocyte itself generates collagen. He mentioned the possibility that some of the collagen is actually adherent to the surface of the hepatocyte and is not freed by the treatment used to measure collagen formation. We know that under certain circumstances liver cells can revert to a more primitive and biochemically polyvalent type, as shown by the activity during regeneration, or during growth in culture. Does the liver cell itself become the producer of the extra fibrous tissue, by the reversion of cells in the damaged liver? Does the reduction of pO_2 as a result of deviation or reduction in blood flow determine a change in the spectrum of primary metabolic activities which the same cell is capable of carrying out?

During these sessions, all of us have emphasized that we need, perhaps as much as anything, a very sharp redefinition of the questions that we are trying to answer and suitable experimental models for answering them. In the course of the meeting we have not come to full agreement on how to achieve this, but I think we did come to certain conclusions. I think we probably agreed that we were wrong in our expectations of being able to differentiate between the consequences of alcohol metabolism and those of alcohol as a drug, by the use of pyrazole or other inhibitors of alcohol dehydrogenase. Most of us hoped that this would be a simple tool, but the complexity that results from use of the pyrazoles is now obvious. We do not simplify or analyse the problem, but instead we contribute to its complexity by the use of such agents. And we are still in a quandary as to how to separate out the various processes that we have referred to. The papers by Drs. DiLuzio, Khanna and Phillips all emphasized that we are dealing with multiple actions that have to be interpreted in terms of drug and alcohol interactions, and which therefore leave us still looking for good experimental models.

Dr. Ugarte, in a round table discussion, emphasized very strongly the question of defining in clinical terms the problems that are encountered, and the need to simulate those properly in our experimental models. This raises the problem of determining the validity of an animal model. It has been pointed out many times during these sessions that the rat, for example, may not be a suitable animal if one is looking for the pathogenesis of the processes that occur in human alcoholic livers. It may be that the failure to produce typical alcoholic hepatitis and Laennec's cirrhosis in the rat is due to limitations in the inherent types of response capability of the rat liver, rather than a

failure of the initiating processes produced by the alcohol itself. It may be that the manner or route of administration, such as the use of a massive single dose of alcohol in the acute fatty liver model, is a poor model if one looks at the patterns of alcohol ingestion by human beings, and the kinds of hepatic pathology that occur, their time relations to the alcohol ingestion, their evolution, and other factors that influence their severity.

In conclusion, it seems to me that during the course of this meeting we *have* reached a consensus on certain main problems in which we are interested, and on certain methods of experimental exploration which we feel have probably not paid off as we had hoped they might. We have also agreed on the need for redefinition of the problems in the search for appropriate models. Finally, I think that we also have now a very much enhanced awareness, due to the epidemiological and clinical data, of the importance of looking at the effects of alcohol itself as a hepatotoxic substance, as one very important factor which, along with metabolic disturbance, physiological disturbance and nutritional disturbance, determines the ultimate evolution of hepatic pathology in the alcoholic human being.

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